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## BACTERIA CONCERNED IN THE PRODUCTION OF THE CHARACTERISTIC FLAVOR IN CHEESE OF THE CHEDDAR TYPE<sup>1</sup>

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In a previous publication (Hastings, Evans, and Hart, 1912)<sup>2</sup> a summary of the knowledge concerning the bacteriology of Cheddar cheese was presented together with the results that were obtained by the authors in an effort to extend the knowledge concerning the flora of this type of cheese. It was shown, as had previously been demonstrated by numerous investigators, that *Bacterium lactis acidi* is an important factor in the making and ripening of Cheddar cheese. The lactic acid formed aids in the curdling of the milk by the rennet, and the acid formed in the curd assists in the expulsion of whey therefrom. The combination of the acid with the paracasein so changes the nature of the curd that the pieces readily coalesce or "mat." The lactic acid also activates the pepsin of the rennet extract, enabling it to act on the paracasein, with the formation of soluble products. The acid reaction of the cheese is maintained during the ripening period and thereafter, thus inhibiting the development of putrefactive bacteria which otherwise would soon destroy the commercial value of the cheese.

It was further shown that lactic bacilli<sup>3</sup> occur in all Cheddar cheese in numbers approximating those of the ordinary lactic bacteria, *B. lactis acidi*, and that coccus forms are also of constant occurrence.

At the beginning of the ripening period the ratio between *Bacterium lactis acidi* and lactic bacilli can, in a general way, be expressed as 99 to 1. This ratio gradually changes as the ripening progresses until in cheese 3 to 6 months old the ratio is reversed. It is apparent that the larger part of the growth of the *B. casei* group must take place after the fermentation

<sup>1</sup> Work of Department of Agriculture in cooperation with Wisconsin Agricultural Experiment Station.

<sup>2</sup> Bibliographic citations in parentheses refer to "Literature cited," pp. 191-192.

<sup>3</sup> This group of lactic-acid-forming organisms appears in the literature under various names, the most common being "lactic bacilli," "*Bacterium bulgaricum*" or "*Bacillus bulgaricus*," and "*Bacterium casei*." The name "*Bacterium casei*" will be used in this article.

of the sugar in the cheese by *B. lactis acidi*. Their continued persistence in large numbers must be due to continued growth for a considerable period or else to their greater resistance to the environment. But little definite information was presented in the bulletin mentioned concerning the occurrence of coccus forms, because of the confusion then existing in the differentiation of some varieties of this group from the *B. lactis acidi* group.

It was recognized that we were not dealing with specific organisms but rather with great groups. In making cheese of the Cheddar type from pasteurized milk it was found that the typical flavor did not develop. The milk, after pasteurization, always contained organisms of the *Bacterium lactis acidi* group, of the *B. casei* group, and coccus forms, and there was added to the milk a starter containing an organism of the *B. lactis acidi* group. In order to obtain a more complete knowledge concerning the biological factors present in Cheddar cheese and their rôle in the development of flavor and possibly to determine the cause of lack of flavor in cheese from pasteurized milk, a more detailed study has been made of cheese both from raw and from heated milk, with the results as presented in this paper.

In continuation of the above-mentioned work, bacteriological analyses of many more cheeses have been made in order to determine more fully the distribution of the groups in ripening cheese; and a detailed study of the pure cultures obtained has been carried out along new lines with the view of correlating the presence of certain types with desirable or undesirable flavor production.

#### METHODS OF EXAMINATION

In the previous work the pure cultures were obtained by isolation from lactose-agar plate cultures and from dilution cultures made in small flasks of sterile milk. These milk cultures were inoculated from a dilution of a cheese emulsion, the dilution increasing from flask to flask by a ratio of 10. The above methods have been modified in the present study. The dilution cultures were made in milk to which was added 1 gram of peptone, 1 gram of dextrose, and 200 c. c. of water per liter. This was found to be more favorable than plain milk for the development of some of the cheese organisms. An effort was made to obtain two pure cultures for study from each series of dilution cultures; viz, the predominating organism of the combined *Bacterium lactis acidi* and coccus groups and the predominating organism of the *B. casei* group. The former was obtained by plating the culture from the highest dilution of the cheese which shows a growth after two days' incubation; the latter was obtained after three weeks' incubation. The flask cultures were then titrated to determine the highest dilution which contained *B. casei*. All milk cultures which titrated more than 1.15 per cent of acidity were further examined for the *B. casei* group. The long incubation usually killed

the other groups of cheese organisms, so that a subinoculation into tubes of litmus milk containing dextrose and peptone resulted in the growth of the lactic bacilli alone. The culture was then purified by plating.

The plate cultures of the cheese dilutions were made in casein agar (Ayers, 1911), to which 1 per cent of dextrose was added; they were incubated at 37° C. for two days and then kept at room temperature for four days. In order to obtain the percentage of the various cheese organisms which develop upon the casein-dextrose-agar plates, a portion containing 10 colonies was circumscribed, and the inclosed colonies were fished off into litmus milk. When growth appeared in the milk cultures a microscopic examination was made. Those cultures from a single plate which caused the same changes in litmus milk and were of the same morphology were considered of the same variety. Representatives of every variety were studied in detail and referred to their respective places, according to the classification given below.

This method of studying only 10 colonies from the plate cultures at one analysis and then reducing this number for more detailed study to a single representative of those cultures which are similar in their morphology and their action on litmus milk was intended to give a broad view of the flora of cheese rather than a detailed one. A more intensive study of the cheeses selected for analysis would necessarily reduce the number which could be examined. Inasmuch as the flora of normal Cheddar cheese will differ greatly in a detailed study, the methods used, which allow the examination of a large number of cheeses and give a rough picture of the cheese flora, were considered the best adapted to increase our knowledge in its present stage.

#### FERMENTATION TESTS FOR THE CLASSIFICATION OF CHEESE ORGANISMS

The cultural characteristics, morphology, and the few biochemical reactions which are ordinarily considered in classification of bacteria were found to be inadequate traits for distinguishing one variety of cheese organism from another of the same group. Therefore the system of classification based upon the fermentation of various test substances which has been used by Gordon (1905) and other English investigators and by Winslow (1912) and his colleagues for the classification of the *Coccaceæ* and which was also used by Rogers and Davis (1912) for classifying the lactic-acid bacteria of the *Bacterium lactis acidi* type was adopted, with modifications suited to the problem in hand, for the classification of cheese organisms.

A sugar-free broth was made of 10 grams of compressed yeast, 10 grams of peptone, and 5 grams of dibasic potassium acid phosphate per liter of water. To this mixture was added 1 per cent of the test substance. At first inoculations were made into dextrose, lactose, galactose, sucrose, salicin, mannit, glycerin, inulin, starch, and raffinose broths with all the

groups of cheese organisms. After many cultures had been submitted to these tests it was found that some of the substances had no differential value. All of the cultures produced acidity from some of the substances, while none of the cultures, or exceedingly few, produced acidity from other substances. Moreover, not all of the test substances which proved of value in differentiating into varieties the members of the coccus and *Bacterium lactis acidii* groups were equally valuable in the study of the *B. casei* group.

After a preliminary study of 250 cultures of the cheese organisms on the 10 test substances with quantitative determinations of the amount of acid produced in the dextrose broth, some of the substances were discarded. The study was continued on 5 of the test substances differing for the various groups of organisms.

It now includes approximately 1,000 cultures of cheese organisms isolated from 37 different raw-milk cheeses, which represent every stage of the ripening period. The test for acid was made after 10 days' incubation at 37° C. Small squares of alkaline litmus paper were placed in the cultures. If the color in several cultures of positive and negative reactions is compared this method will detect very slight increases in acidity.

This method of testing the production of acid with litmus paper differs from that of other investigators who have made use of the fermentation tests for the classification of bacteria. These investigators have determined by titration the amount of acid produced in the various broths and have considered these data also in their classification. But in any work of the nature of the problem under discussion it is important that the first stages should be a comprehensive survey of all of the factors which may influence the final result rather than a more intensive study of any one or more factors which attract attention at first sight. The methods which have been followed in the classification of cheese organisms were chosen with the purpose of excluding as much as possible of the routine work, which appeared to be of minor importance for this study in its present stage, in order to extend it over a larger number of samples of cheese. For this reason the fermentable substances employed for the tests were reduced to the smallest number which appeared adequate for the differentiation. And the titration of the acid produced, which in the preliminary study was made in the dextrose broth alone, was entirely omitted in the major part of the study. Rogers and Davis (1912) have adopted an increase of 1 per cent of normal acid as the division between fermentation and nonfermentation. In the case of lactose, salicin, sucrose, mannit, and inulin the reddening of alkaline litmus paper gives practically the same results as far as the division into fermenters and nonfermenters is concerned, for in broths containing these substances the reaction is almost always negative or decidedly positive, with the development of a considerable acidity. If there is a development of acidity in glycerin, the reaction is often slight. The titration of a num-

ber of such cultures showed that the litmus paper will detect increases of acidity as low as 0.5 per cent; these were recorded as positive. Hence, in the comparatively few instances in which the development of acid is slight the results may sometimes be recorded as positive in this study, where other investigators would have recorded them as negative.

The amount of acid produced is regarded as unimportant in the classification of the cheese organisms. The consideration of degrees of acidity would greatly complicate a system which, if it is to be valuable in the study of the cheese problem, should rather be made as simple as possible.

#### CLASSIFICATION OF THE *BACTERIUM LACTIS ACIDI* AND COCCUS GROUPS

In the present study an especial effort has been made to differentiate the *Bacterium lactis acidi* and coccus groups. Since they were submitted to the same tests, the classification of these groups will be considered together. The organic substances which were adopted as the most useful test substances for the coccus and *B. lactis acidi* groups are lactose, salicin, mannit, sucrose, and glycerin. The morphology, the growth on plain agar slopes, and the growth in litmus milk were also considered.

Cultures in which some or all of the cells are elongated and in pairs and which curdle litmus milk with the reduction of the litmus characteristic for *Bacterium lactis acidi* were classed in that group. The Streptococcus group includes all cultures in which the cells are spherical and arranged in pairs or in chains. The cultures of this group do not give the reduction of litmus which is characteristic for the *B. lactis acidi* group. Most of the cultures of the Micrococcus group produce a heavy growth upon the agar slope, which is often of some shade of yellow. They are the members of the Coccaceæ which divide in two planes; consequently the cells appear in pairs, fours, or bunches.

In Table I the sign  $\pm$  indicates that individual cultures of the given variety may produce acid from the substance in question; or they may fail to produce acid. The test substances are arranged in the table according to their order of availability. For example, lactose is fermented by the greatest number of cultures, and mannit is fermented by the least number of cultures of both groups of the Coccaceæ.

TABLE I.—Classification of the groups of cheese organisms

Variety No.	Production of acidity in broth containing—				
	Lactose.	Salicin.	Mannit.	Sucrose.	Glycerin.
a	+	—	—	—	—
b	+	+	—	—	—
c	+	+	—	+	—
d	+	$\pm$	+	$\pm$	—

TABLE I.—Classification of the groups of cheese organisms—Continued

## STREPTOCOCCUS

Variety No.	Production of acidity in broth containing—				
	Lactose.	Salicin.	Sucrose.	Glycerin.	Mannit.
a.....	—	—	—	—	—
b.....	+	—	±	±	—
c.....	+	+	±	±	—
d.....	+	±	±	±	+

## MICROCOCOCCUS

Variety No.	Production of acidity in broth containing—				
	Lactose.	Sucrose.	Glycerin.	Salicin.	Mannit.
a.....	+	±	±	—	—
b.....	+	±	±	+	—
c.....	+	+	—	—	+

All organisms which have been found constantly in cheese are included in the four groups *Bacterium lactis acidi*, Streptococcus, and Micrococcus groups, together with the *B. casei* group, which will be discussed later. The different varieties of all four groups produce acid in milk. Spore-forming organisms occur in very small numbers or are entirely absent. The same thing is true of liquefying organisms. Whenever they are present in sufficient numbers to have an appreciable influence in the ripening, it is to the detriment of good flavor.

In the classification of the *Bacterium lactis acidi* group, variety "a" corresponds to group "A" in Rogers and Davis's classification of the lactic-acid bacteria (1912). Varieties "b," "c," and "d" are all included in their group "B," which also includes other varieties which were not found among the cheese organisms and are therefore not mentioned in this work.

#### CONSTANCY OF REACTION OF THE CULTURES OF THE BACTERIUM LACTIS ACIDI AND COCCUS GROUPS TO THE TEST SUBSTANCES

It is obvious that the accuracy of this method, in so far as it is a guide for the classification of an individual culture, depends on the constancy of the reactions to the tests. It is the opinion of those who have made a comprehensive study of this method that the fermentation reactions on the test substances are on the whole remarkably constant for any given strain, and therefore they are of value in classification. Representative cultures of the varieties of the coccus and the *Bacterium lactis acidi*

groups have been maintained on agar slopes, with transfers made every month or six weeks. The constancy of the reactions of these cultures on the five organic substances has been tested, and the results on salicin, mannit, sucrose, and glycerin are given in Table II. The results on lactose are omitted, because they are always positive.

TABLE II.—The constancy of the *Dacterium lactis acidii* and *coccus* groups in their action upon test substances.

BACTERIUM LACTIS ACIDI														
No. of culture.	Production of acid in broth containing—													
	Salicin.				Mannit.			Sucrose.			Glycerin.			
	First test.	Age of culture at time of second test.	Sec. test.	Age of culture at time of third test.	Third test.	First test.	Sec. test.	Third test.	First test.	Sec. test.	Third test.	First test.	Sec. test.	Third test.
	—	Months.	—	Months.	—	—	—	—	—	—	—	—	—	—
1.....	—	8	—	12	—	—	—	—	—	—	—	—	—	—
2.....	—	3	+	12	+	—	+	—	—	—	—	—	—	—
3.....	—	9	+	12	+	—	+	—	—	—	—	—	—	—
4.....	+	1	+	—	—	—	—	—	—	—	—	—	—	—
5.....	+	4	+	8	+	—	—	—	—	—	—	—	—	—
6.....	—	4	+	8	+	—	+	—	—	—	—	—	—	—
7.....	+	4	+	8	+	—	+	—	—	—	—	—	—	—
8.....	+	2	+	—	—	—	—	—	—	—	—	—	—	—
STREPTOCOCCUS														
No. of culture.	Production of acid in broth containing—													
	Salicin.				Sucrose.			Glycerin.			Mannit.			
	First test.	Age of culture at time of second test.	Sec. test.	Age of culture at time of third test.	Third test.	First test.	Sec. test.	Third test.	First test.	Sec. test.	Third test.	First test.	Sec. test.	Third test.
	—	Months.	—	Months.	—	—	—	—	—	—	—	—	—	—
9.....	—	10	—	14	—	—	+	—	—	—	—	—	—	—
10.....	—	10	—	14	—	—	+	—	—	—	—	—	—	—
11.....	—	6	—	—	—	—	+	—	—	—	—	—	—	—
12.....	—	5	—	—	—	—	+	—	—	—	—	—	—	—
13.....	—	9	—	—	—	—	+	—	—	—	—	—	—	—
14.....	+	9	—	12	+	—	—	—	—	—	—	—	—	—
15.....	+	9	—	8	+	—	—	—	—	—	—	—	—	—
16.....	+	4	+	8	+	—	+	—	—	—	—	—	—	—
17.....	+	5	+	—	—	—	+	—	—	—	—	—	—	—
18.....	—	6	—	9	—	—	—	—	—	—	—	—	—	—
19.....	+	5	+	9	—	—	+	—	—	—	—	—	—	—
20.....	+	4	+	8	+	—	+	—	—	—	—	—	—	—



TABLE II:—The constancy of the *Bacterium lactis acidii* and coccus groups in their action upon test substances—Continued

Micrococcus														
No. of culture.	Production of acid in broth containing—													
	Sucrose.				Glycerin.			Salicin.			Mannit.			
	First test.	Age of culture at time of second test.	Second test.	Age of culture at time of third test.	Third test.	First test.	Second test.	Third test.	First test.	Second test.	Third test.	First test.	Second test.	Third test.
		Months.		Months.										
21.....	—	8	—	11	+	—	—	—	—	—	—	—	—	—
22.....	—	10	+	14	—	—	—	—	—	—	—	—	—	—
23.....	+	9	+	13	+	—	—	—	—	—	—	—	—	—
24.....	+	9	+	13	+	—	—	—	—	—	—	—	—	—
25.....	+	6	—	9	+	—	—	+	—	—	—	—	—	—
26.....	—	6	—	9	+	—	—	+	—	—	+	—	—	—
27.....	+	4	+	8	+	—	—	+	—	—	+	—	—	—

In the *Bacterium lactis acidii* group there is a tendency for those cultures which have a low ability for fermentation to increase that ability when cultivated upon the plain agar slopes. This appears to be a change in the physiological properties of the cultures when grown under artificial conditions, for every variation which occurred in this group was an increased ability to attack the more difficultly fermentable substances. Thus, cultures 2 and 3, which at the time the first test was made fermented only lactose and were classified as variety "a," in later tests fermented also salicin and mannit and would therefore be classified as variety "d." Also, culture 5 acquired the ability to ferment mannit, and culture 6 acquired the ability to ferment salicin.

The Streptococcus cultures all remained constant in their action upon lactose, salicin, and mannit. Cultures 9 and 10 acquired the ability to ferment sucrose, and culture 10 also acquired the property of fermenting glycerin. Culture 19 lost the power of fermenting glycerin. The Micrococcus cultures also showed some inconstancy upon sucrose and glycerin, and one culture, 25, varied in its action on salicin. On the whole, the reactions are sufficiently constant to be considered valuable in the differentiation of cheese organisms.

#### CLASSIFICATION OF THE BACTERIUM CASEI GROUP BY BIOCHEMICAL TESTS

The study of a number of cultures of the *Bacterium casei* group isolated from Cheddar cheese showed that there is a great variation in the amount of acidity the different cultures are able to produce in milk. It has been found (Currie, 1911) also that the cultures differ in the rotatory power of their acids. It was evident that different strains of this group were present in cheese, and therefore it was desirable to classify them, in order to study the significance of the various strains in cheese ripening. Accordingly the biochemical tests which were used for the differentiation of the other cheese organisms were adopted also for the classification of this group.

The preliminary study upon the 10 original test substances showed that lactose, salicin, sucrose, mannit, and inulin have some value in the differentiation of this group. The other five test substances were discarded as of too little value to compensate for the time required for their use.

The morphology of this group differentiates it from the other cheese organisms.

Two hundred and forty-nine cultures of the *Bacterium casei* group have been submitted to the tests, which differentiated them into 12 strains, as given in Table III. It was necessary to combine this large number of strains into varieties, in order to make the classification workable in the study of the cheese problem.

TABLE III.—Types of *Bacterium casei* as differentiated by biochemical tests

Strain No.	Number of cultures studied.	Production of acid in broth containing—				
		Lactose.	Salicin.	Sucrose.	Mannit.	Inulin.
1.....	5	—	—	—	—	—
2.....	117	+	—	—	—	—
3.....	22	+	+	—	—	—
4.....	12	+	—	+	—	—
5.....	18	+	+	+	—	—
6.....	7	+	—	—	+	—
7.....	14	+	+	—	+	—
8.....	7	+	—	+	+	—
9.....	28	+	+	+	+	—
10.....	1	+	—	+	—	+
11.....	12	+	+	+	—	+
12.....	7	+	+	+	+	+

Strain 2, which ferments lactose alone and includes almost one-half of the cultures studied, naturally formed one variety. With it was included the five cultures which failed to ferment lactose in broth cultures, although they fermented lactose in milk cultures. The second variety includes all cultures which ferment lactose, and one, two, or three of the test substances—salicin, sucrose, and mannit. A third variety is characterized by its ability to ferment inulin. This variety also ferments lactose and one, two, or three of the substances, salicin, sucrose, and mannit. The three varieties have been designated "a," "b," and "c" in Table IV.

TABLE IV.—Varieties of *Bacterium casei* found in cheese

Variety No.	Production of acid in broth containing—				
	Lactose.	Salicin.	Sucrose.	Mannit.	Inulin.
a.....	+	—	—	—	—
b.....	+	±	±	±	—
c.....	+	±	±	±	+

There is a great variation in the amount of acidity formed in milk by the various cultures of a given strain. In Table V are given the range of maximum percentages of acidity found within the strain. The average percentage of acidity formed by the cultures increases with the ability to break down the more complex test substances. The average percentages for the three varieties are given in Table VI. The acid has been calculated as lactic acid.

TABLE V.—Range of percentages of acidity formed by the strains of *Bacterium casei*

Strain No.	Number of cultures studied.	Range of maximum percentages of acidity.	Strain No.	Number of cultures studied.	Range of maximum percentages of acidity.
1.....	1	0.68	7.....	12	0.91 to 1.97
2.....	60	.10 to 1.52	8.....	6	.94 to 1.69
3.....	8	.73 to 2.37	9.....	14	1.38 to 1.80
4.....	12	.42 to 1.50	10.....	1	.97
5.....	16	.47 to 1.97	11.....	6	.86 to 1.60
6.....	5	.85 to 1.35	12.....	1	1.74

TABLE VI.—Variation in the average percentage of acidity formed by the varieties of *Bacterium casei*

Variety No.	Number of cultures titrated.	Average percentage of acidity.
a.....	60	0.75
b.....	72	1.03
c.....	10	1.23

The statement was made in a previous publication (Hastings, Evans, and Hart, 1912) that the width of the cells of the lactic bacilli in a pure culture appear to be fairly constant, but that in some cultures the individual cells are all slender, whereas in other cultures they are all comparatively thick. Measurements have been made of the width of the cells in many cultures. The averages for the three varieties are given in Table VII.

TABLE VII.—Variations in the average width of the cells of the varieties of *Bacterium casei*

Variety No.	Number of cultures measured.	Average width of the cells in microns.
a.....	25	1.06
b.....	22	.81
c.....	3	.53

Thus, it is shown that the width of the cells decreases with their ability to attack the more difficultly fermentable substances and with their ability to produce higher percentages of acidity. The differences in width are so great that with a glance at a microscopic field of a culture containing

cells of either extreme in width, the culture may be classed as a high or a low acid-producing variety.

#### DISTRIBUTION OF THE GROUPS AND VARIETIES OF CHEESE ORGANISMS IN NORMAL CHEDDAR CHEESE

Twenty-one raw-milk cheeses have been analyzed bacteriologically and the cultures classified in the manner described above. Some of the cheeses were analyzed only once or a few times. In Table VIII are given the percentages of the different varieties which were found in a series of 16 cheeses. These were the best of a large number of cheeses which had been sent for scoring to the Dairy Department of the University of Wisconsin from cheese factories in different parts of the State. They represent a high quality of Wisconsin cheese.

It will be observed that almost every variety of each of the four groups of cheese organisms is represented in the 16 cheeses in sufficiently large numbers to be influential in the development of flavor. It is not to be supposed that representatives of all of the varieties occurring in a cheese can be isolated each time the cheese is submitted to analysis. Particularly in the case of those cheeses which were analyzed only once, other forms than those shown by the data must have been present. For this reason Table VIII as a whole rather than the data from any individual cheese should be considered in drawing conclusions as to the types of organisms present in normal cheese. The figures of the table indicate that the flora of a normal Cheddar cheese is varied and consists of representatives of each of the four groups of cheese organisms.

TABLE VIII.—Bacterial content of normal raw-milk cheeses

Cheese No.	Age.	Bacteria per gram (plate cultures).	Variety of Bacterium lactis acid.			Variety of Bacterium casei.			Variety of streptococci.			Variety of micrococci.			Bacteria per gram (dilution cultures).	Variety of Bacterium casei.	
			a b d			a b c			a b c d			a b c				a	b
			P.c.	P.c.	P.c.	P.c.	P.c.	P.c.	P.c.	P.c.	P.c.	P.c.	P.c.	P.c.			
33.....	Days.	30	35,000,000	.....	100	.....	.....	.....	.....	.....	.....	.....	.....	100,000,000	.....	+	.....
35.....	30	8,000,000	.....	63	.....	25	.....	.....	.....	.....	.....	.....	.....	10,000,000	.....	+	.....
44.....	31	4,000,000	.....	25	.....	.....	.....	.....	.....	.....	.....	.....	.....	10,000,000	.....	+	.....
36.....	28	10,500,000	.....	40	.....	10	20	.....	.....	.....	.....	.....	.....	1,000,000,000	.....	+	.....
40.....	31	3,200,000	.....	.....	.....	71	29	.....	.....	.....	.....	.....	.....	10,000,000	.....	+	.....
38.....	48	2,000,000	.....	12	.....	13	12	.....	.....	.....	.....	.....	.....	10,000,000	.....	+	.....
45.....	38	55,000,000	.....	67	.....	.....	.....	.....	.....	.....	.....	.....	.....	1,000,000	.....	+	.....
50.....	24	7,000,000	.....	30	.....	50	.....	.....	.....	.....	.....	.....	.....	100,000,000	.....	+	.....
53.....	30	8,000,000	.....	70	.....	.....	.....	.....	.....	.....	.....	.....	.....	10,000,000	.....	+	.....
63.....	30	3,300,000	.....	10	30	50	.....	.....	.....	.....	.....	.....	.....	10,000,000	.....	+	.....
45.....	15	300,000,000	.....	90	.....	.....	.....	.....	.....	.....	.....	.....	.....	100,000,000	.....	+	.....
5.....	52	16,000,000	.....	11	.....	.....	.....	.....	.....	.....	.....	.....	.....	10,000,000	.....	+	.....
.....	68	11,000,000	.....	20	.....	.....	.....	.....	.....	.....	.....	.....	.....	10,000,000	.....	+	.....
.....	37	28,000,000	.....	50	.....	.....	.....	.....	.....	.....	.....	.....	.....	100,000,000	.....	+	.....
13.....	43	25,000,000	.....	63	.....	.....	.....	.....	.....	.....	.....	.....	.....	100,000,000	.....	+	.....
59.....	31	31,000,000	.....	.....	.....	30	10	46	.....	.....	.....	.....	.....	1,000,000	.....	+	.....
.....	39	10,000,000	.....	14	.....	.....	.....	.....	.....	.....	.....	.....	.....	10,000,000	.....	+	.....
46.....	50	5,000,000	.....	14	.....	.....	.....	.....	.....	.....	.....	.....	.....	10,000,000	.....	+	.....
.....	62	4,500,000	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	10,000,000	.....	+	.....
12.....	12	12,000,000	.....	90	.....	.....	.....	.....	.....	.....	.....	.....	.....	10,000,000	.....	+	.....
.....	25	15,000,000	.....	50	.....	.....	.....	.....	.....	.....	.....	.....	.....	100,000,000	.....	+	.....
.....	41	17,500,000	.....	10	.....	.....	.....	.....	.....	.....	.....	.....	.....	1,000,000	.....	+	.....
.....	16	600,000,000	.....	.....	.....	17	83	.....	.....	.....	.....	.....	.....	100,000,000	.....	+	.....
73b.....	39	15,000,000	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1,000,000,000	.....	+	.....
.....	14	58,000,000	.....	76	.....	.....	.....	.....	.....	.....	.....	.....	.....	100,000,000	.....	+	.....
53.....	30	130,000,000	.....	11	.....	.....	.....	.....	.....	.....	.....	.....	.....	100,000,000	.....	+	.....

+ = Found present.

A more comprehensive study was made of raw-milk cheese No. 17 R. Analyses were made during the making and at intervals throughout the ripening period until the cheese was 8 months old. The results of this study are given in Table IX.

TABLE IX.—*Bacterial content of raw-milk cheese No. 17 R*

Age.	Bacteria per gram (plate cultures).	Variety of <i>Bacterium lactis acid.</i>				Variety of <i>Bacterium casei</i> .				Variety of streptococci.				Variety of micrococci.		Bacteria per gram (dilution cultures).	Variety of <i>Bacterium lactis acid.</i>				Variety of <i>Bacterium casei</i> .	
		a	b	c	d	a	b	c	d	a	b	c	d	a	b		a	b	c	d	a	b
Days.																						
Card.																						
100,000,000	30.0															100,000,000						
70,000,000	70.0															100,000,000	99.999					0.001
40,000,000	10.0															1,000,000,000						0.01
20,000,000	88.8									11.2						1,000,000,000	9.99					1.00
15,000,000	50.0									10						100,000,000	99.0					1.0
10,000,000	27.5					12.5										100,000,000	99.0					1.0
5,000,000	23.2									11.9	0.3					100,000,000	99.0					1.0
2,000,000	10.0					20.0										100,000,000	90.90					9.1
1,000,000	43.0					56.3				2	2			0.3		100,000,000	90.9					9.1
500,000	70.0									17.5				12.5		100,000,000	90.9					9.1
250,000	26.5									30.0	3.5					100,000,000	90.9					9.1
125,000	88.8									10.0	1.2					100,000,000	90.0					9.9
62,500	41.7					20.0								3.2		100,000,000	90.9					9.1
31,250	10.0									10.0				3.1		100,000,000	99.0					1.0
15,625	27.0													11.0		100,000,000	90.9					9.1
7,812	30.0															100,000,000	50.0					50.0

Where very small percentages appear in the columns of the coccus groups the figures were obtained by plating upon an agar made with cheese extract. Upon this medium the lactic-acid groups of organisms form very small colonies, but the cocci, particularly the micrococci, develop large colonies and can be easily differentiated and isolated from plates which are very thickly seeded.

Cheese No. 17 R was a good Cheddar cheese, with the characteristic flavor well developed when 1 month old. The cheese remained in good condition until the last analysis, with an increasing sharpness of taste which is common to well-matured cheese. The flora is considered typical of a good Cheddar cheese. The data confirm those presented in Table VIII. Twelve of the fifteen varieties of cheese organisms representing the four groups were isolated from this cheese. The data presented in Tables VIII and IX show that besides the *Bacterium lactis acidii* group, which has long been known to be important in cheese ripening, and the *B. casei* group, which was shown (Hastings, Evans, and Hart, 1912) to be constantly present in cheese in numbers almost as great as the former group. Coccus forms are also constantly present, and although they are fewer in number than the lactic-acid-forming groups they occur in sufficient numbers to affect flavor development.

Other evidence of the constant presence of several varieties of bacteria in the cheese flora is found in the gradual decline in acid production, as the dilutions increase, in milk inoculated with the cheese emulsion. In the present study of cheese flora, several hundreds of series of titrations

have been made of the milk cultures which have been mentioned as a part of the routine method of cheese analysis. There is in nearly every series a general decline as the emulsion used for the inoculation becomes more and more dilute. The two series presented in Table X illustrate the point. The titrations were made after 28 days incubation at 35° C. and the acidity calculated as lactic acid.

TABLE X.—Decline in acidity with increasing dilutions of cheese emulsion used for inoculation of milk cultures

Cheese No. 372 C.		Cheese No. 309 C.	
Dilution.	Percentage of acidity.	Dilution.	Percentage of acidity.
1:100,000.....	1.60	1:100,000.....	1.54
1:1,000,000.....	1.55	1:100,000.....	1.45
1:10,000,000.....	1.22	1:1,000,000.....	1.50
1:100,000,000.....	.78	1:10,000,000.....	1.18
1:1,000,000,000.....	.67	1:100,000,000.....	1.00
1:10,000,000,000.....	(a)	1:1,000,000,000.....	(a)

<sup>a</sup> No growth.

The lower percentage of acidity with every tenfold dilution indicates that in each dilution there were present certain varieties of organisms in such small proportions that they did not occur in the next higher dilution. Out of 345 series of titrations the decline was without a break in 56 per cent of the cases; in 37 per cent of the cases there was a general decline, but with one or more breaks; in only 7 per cent of the cases was this decline not apparent.

#### THE VARIATION IN CHEESE FLORA

It is evident from Tables VIII and IX that the normal flora of Cheddar cheese is varied, with varieties of all four groups of cheese organisms in numbers ranging from hundreds of thousands to billions per gram of cheese. The flora in two equally good cheeses will contain each of the four groups of cheese organisms, but will differ as to the varieties present. There will also be a variation in the proportion of organisms of any single group. It does not appear that it is essential to good flavor to have any single variety present in very high proportions. One cheese may contain a given variety in high proportions, whereas another cheese may have this variety in such low proportions that it does not make its appearance in a bacteriological analysis. To illustrate: *Streptococcus* "b," which formed 30 per cent of the flora of cheese No. 36 (Table VIII), was isolated from only 6 of the 17 cheeses whose flora are presented in Tables VIII and IX.

This variation of flora, with a constancy to the general type of growth, is to be expected under any ecological conditions determined by natural

circumstances, for the ripening of Cheddar cheese must be regarded as a spontaneous fermentation, limited to certain groups of bacteria by the conditions obtaining in the cheese mass and subject to variation within the groups by accident of inoculation before or during the cheese making, or by slight variations in the raw material or in the method of manufacture, which enable one variety or the other to gain an ascendancy.

#### SYMBIOTIC RELATION OF CHEESE ORGANISMS

The complex chemical changes taking place in a mixed culture of bacteria have usually been regarded as the result of the combined activity of the individual properties of each of the several species when grown alone, with these properties accelerated in some cases by the associated action.

Among the many cultures of cheese organisms inoculated into the carbohydrate media, it was observed that when by accident the culture was contaminated with other cheese organisms through failure of a pure isolation, there appeared to be a high ability to attack the more complex substances. Accordingly many inoculations of cheese cultures were made into the test media in various combinations, to determine whether associative action would give new properties to the cultures concerned. In this experiment the test for acid production was the reddening of litmus paper. In many cases acid was produced from a given substance by the associated action, when neither culture working alone would give such a reaction. A few examples are given in Table XI to show the results of these inoculations.

TABLE XI.—The effect of the associative action of cheese organisms in breaking down test substances

Organism.	Production of acid in broth containing—					
	Lactose.	Salicin.	Sucrose.	Glycerin.	Mannit.	Inulin.
Bacterium lactis acidi, a.....	+	—	—	—	—	—
Bacterium casei, a.....	+	—	—	—	—	—
Bacterium casei, b.....	+	+	—	—	+	—
Streptococcus, b.....	+	—	—	—	—	—
Streptococcus, c.....	+	+	+	—	—	—
Micrococcus, b.....	+	—	—	—	—	—
Bacterium lactis acidi, a; Bacterium casei, a.....	+	—	+	—	—	—
Bacterium lactis acidi, a; Bacterium casei, b.....	+	+	+	—	+	—
Bacterium lactis acidi, a; Streptococcus, b.....	+	+	+	—	—	—
Bacterium lactis acidi, a; Streptococcus, c.....	+	+	+	+	—	—
Bacterium casei, a; Bacterium casei, b.....	+	+	+	—	+	—
Bacterium casei, a; Streptococcus, b.....	+	—	+	—	—	—
Bacterium casei, a; Streptococcus, c.....	+	+	+	+	+	—
Bacterium casei, a; Micrococcus, b.....	+	+	+	—	—	—

Further inoculations were made into broth containing the test substances to determine the quantitative effect of this associative action. The results of some of these inoculations are given in Table XII, in which the acidity is expressed as lactic acid.

TABLE XII.—The effect of the associative action of cheese organisms in the production of acid

Organism.	Medium.					
	Salicin.		Sucrose.		Mannit.	
	Individual action.	Associative action.	Individual action.	Associative action.	Individual action.	Associative action.
	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.
<i>Bacterium lactis acidii</i> , a.....	0.00	0.00	0.00	0.00	0.00	0.00
<i>Bacterium casei</i> , a.....	0.00	0.39	0.00	0.00	0.00	0.00
<i>Bacterium lactis acidii</i> , a.....	0.00	0.00	0.00	0.00	0.00	0.00
<i>Streptococcus</i> , b.....	0.00	.12	0.00	0.00	0.00	0.00
<i>Bacterium lactis acidii</i> , b.....	0.00	0.00	0.00	0.00	0.00	0.00
<i>Bacterium casei</i> , a.....	.30	.37	0.00	.28	0.00	0.00
<i>Bacterium lactis acidii</i> , b.....	0.00	0.00	0.00	0.00	0.00	0.00
<i>Micrococcus</i> , c.....	.39	.32	.44	.51	0.00	.35
<i>Bacterium lactis acidii</i> , d.....	0.00	0.00	0.00	.17	0.00	.28
<i>Bacterium casei</i> , a.....	0.00	0.00	0.00	0.00	0.00	0.00
<i>Bacterium lactis acidii</i> , d.....	0.00	0.00	0.00	0.00	0.00	0.00
<i>Micrococcus</i> , b.....	0.00	.21	0.00	0.00	.33	.31

The data show that considerable quantities of acid can be formed in mixed cultures even though the individual cultures are unable to ferment the substance in question. In the *Bacterium lactis acidii* group, 13 positive reactions were obtained and determined by titration in cultures in which a member of this group was mixed with a representative of one of the other three groups of cheese organisms, while 23 cases were negative. In the entire 36 tests none of the organisms was able to ferment the substance used when working alone. The same symbiotic relations were exhibited by the *B. casei* group when a member of this group was grown together with a representative of one of the other groups of cheese organisms or with another variety of the same group. (See Table XI.) Thus, it appears not only that the influence upon each other of the cheese organisms is beneficial in enhancing the individual powers in many cases but also that the symbiosis enables the cheese organisms to attack other food substances than any one species working alone would be able to utilize.

The fact that the normal flora of Cheddar cheese consists of a number of varieties of the four groups of cheese organisms and that these exert upon one another a decided influence in their chemical activities is most important in considering the cheese-ripening problem.



## BACTERIAL FLORA OF PASTEURIZED MILK CHEESE

The influence of this varied flora upon the production of flavor is emphasized in a study of the flora of pasteurized-milk cheese. During the pasteurization approximately 99 per cent of the bacteria of the milk are killed (Sammis and Bruhn, 1912); then there are added, in the form of a starter, organisms belonging to the *Bacterium lactis acidii* group. But instead of ripening the milk until the desired acidity for cheese making is obtained—the usual procedure in the making of raw-milk cheese—a weak solution of hydrochloric acid is added to make up the required acidity. This is necessary in preparing pasteurized milk for cheese making.

It is evident that the initial bacterial flora of the cheese made from pasteurized milk must differ from the flora of a raw-milk cheese of the same age, for in the latter all of the types of organisms making up the varied flora of milk have had an opportunity to develop without any other restraint than that of their symbiotic relations with the associated types.

In studying the flora of cheese in respect to its influence in flavor production many bacterial analyses have been made of pasteurized-milk cheese in every stage of the ripening process. The cultures isolated have been studied in the same manner as those from raw-milk cheese and have been classified accordingly. In Tables XIV and XV are presented the results of the analyses of two pasteurized-milk cheeses, Nos. 20 and 21, to which pure-culture starters were added. To cheese No. 20 (Table XIII), there was added 0.75 per cent of a pure-milk culture of *Bacterium lactis acidii*, b. In striking contrast to the varied flora of raw-milk cheese, as presented in Tables VIII and IX, it will be observed that the bacterial flora of this cheese consisted almost entirely of the one variety of organism, *B. lactis acidii*, b, which was added to the milk for a starter. Only once was another variety of this group isolated.

TABLE XIII.—Bacterial content of pasteurized-milk cheese No. 20 to which was added 0.75 per cent of a culture of *Bacterium lactis acidii*, b

Age.	Plate cultures.						Dilution cultures.					
	Bacterial content.	Variety of <i>Bacterium lactis acidii</i> .		Variety of streptococci.	Variety of micrococci.		Confirmation.	Bacterial content.	Variety of <i>Bacterium lactis acidii</i> .	Variety of <i>Bacterium casei</i> .		
		a	b		a	b				a	b	
<i>Days to state.</i>												
Milk..	9,600,000	P. d.	P. d.	P. d.	P. d.	P. d.	P. d.	100,000,000	P. d.	P. d.	P. d.	0.000
Curd..	20,000,000		95-70				0-30	100,000,000	99-9999	0.0001		0.000
6....	450,000,000		98-00				2-00	100,000,000	99-9999	0.0001		0.000
14....	175,000,000		95-50				3-00	100,000,000	100-00			
22....	175,000,000		98-30				1-70	100,000,000	100-00			
30....	400,000,000		95-75				4-15	10,000,000,000				
38....	300,000,000	28-2	70-50				1-30	1,000,000,000				
47....	1,650,000,000		100-00					1,000,000,000	99-00	1-0000		
56....	65,000,000		99-77	0-04		0-10						
78....	170,000,000		100-00					100,000,000	90-90			9-100

One variety of the *Streptococcus* group was isolated once, and two varieties of the *Micrococcus* were isolated, each only once, in small percentages. The small percentages of cocci were isolated from cheese-agar plates in the same manner as was followed in the analysis of the raw-milk cheese presented in Table IX. Cheese No. 20 was quite badly contaminated with a liquefying coccus which infected each of the three vats of milk made up separately into cheese. The contamination is given in a separate column. The data from the dilution cultures show *Bacterium lactis acidii*, b, as the predominating organism of the cheese at every examination. The *B. casei* group developed slowly in this cheese. It was present in exceedingly small percentage during the first few days. Unfortunately the dilutions were made too high, so that this group did not appear in the dilution cultures in the analyses made between the sixth and forty-seventh days. At the forty-seventh and seventy-second days there were present 1,000,000 bacteria of this group per gram of cheese.

TABLE XIV.—Bacterial content of pasteurized-milk cheese No. 21 to which was added 0.75 per cent of a culture of *Bacterium lactis acidii*, d

Age.	Plate cultures.				Dilution cultures.			
	Bacteria content.	Variety of <i>B. lactis acidii</i> , d.	Variety of micrococci.		Bacterial content.	Variety of <i>B. lactis acidii</i> , d.	Variety of <i>Bact. casei</i> .	
			a	b			a	b
Days or state.	Per cent.	Per ct.	Per ct.		Per ct.	Per ct.	Per ct.	
Milk					10,000,000	100.00		
Curd	250,000,000	100.00			100,000,000			
5	270,000,000	100.00			100,000,000	99.999	0.001	
13	1,120,000,000	100.00			1,000,000,000	100.00		
21	2,200,000,000	100.00			1,000,000,000			
29	250,000,000	100.00			1,000,000,000	100.00		
37	1,400,000,000	99.99	0.01		1,000,000,000			
45	1,900,000,000	99.99	0.01		1,000,000,000	99.99	0.001	
53	1,000,000,000	99.94	0.02	0.04				
71	1,430,000,000	100.00			1,000,000,000	99.90		0.1

Cheese No. 21 (see Table XIV) received three-fourths per cent of *Bacterium lactis acidii*, d, as a starter. A study of the pure cultures isolated from this cheese shows that this variety composed practically 100 per cent of the cheese organisms, as determined by plate cultures. No other variety of *Bacterium lactis acidii* and no variety of the *Streptococcus* group appeared. Small percentages of micrococci were isolated on the thirty-seventh, forty-sixth, and fifty-seventh days. The pure cultures isolated from the dilution flasks showed only *B. lactis acidii*, d, and a slowly increasing number of *B. casei*, which reached 1,000,000 of these bacteria per gram of cheese on the forty-sixth day.

The data in Tables XIII and XIV illustrate well the facts which are demonstrated by a study of the results of many analyses of 14 pasteurized-milk cheeses. In some of these cheeses a mixture of several

pure cultures was used as a starter. Usually every type which was added to the cheese was isolated in considerable percentages in some one or more of the analyses. Thus, it has been shown that the flora of pasteurized-milk cheese consists almost entirely of the organisms which are introduced in the starter, with a small percentage of micrococci and steadily increasing numbers of *Bacterium casei*.

#### RELATION OF THE VARIOUS TYPES OF CHEESE ORGANISMS TO FLAVOR PRODUCTION

Since it has been shown that in pasteurized-milk cheese the bacterial content is practically limited to the varieties which are added during the making, together with small percentages of micrococci, and a development of the *Bacterium casei* group similar to that in a raw-milk cheese, although it is usually more or less retarded, it is possible to gain some knowledge of the influence of the different varieties in pure culture upon flavor production.

A normal raw-milk cheese of the Cheddar type will, after a few days' ripening, begin to develop a delicate flavor which is characteristic of this type of cheese. This flavor becomes intensified as the cheese matures, and after ripening for a number of weeks, depending upon the temperature at which the cheese is kept, it acquires a pungent taste which also intensifies with continued ripening.

A pasteurized-milk cheese made with a commercial starter will never develop the Cheddar flavor which characterizes a young raw-milk cheese of this type, but it does develop an acid flavor which is pleasant to the taste of many people. If the cheese is kept for several months, the acid flavor disappears and the biting taste common to well-matured raw-milk cheese becomes the characteristic flavor.

#### THE RÔLE OF BACTERIUM CASEI IN FLAVOR PRODUCTION

The floras of the two types of cheese, which differ essentially during the first few weeks of ripening, become more and more alike as the cheese matures. As shown in Table XV, *Bacterium casei* develops in pasteurized-milk cheese in as large numbers as in raw-milk cheese. It is reasonable, therefore, to ascribe to this group of organisms the development of the pungent flavor common to the two types of well-matured cheese.

In Table XV are given the numbers of *Bacterium casei* in a raw-milk and in a pasteurized-milk cheese made the same day from the same lot of milk. Until the forty-second day the raw-milk cheese contains about 10 times as many of the *B. casei* as the pasteurized-milk cheese, but after the forty-second day the cheeses contain about equal numbers of these organisms.

Cheeses Nos. 307 C and 307 illustrate the *Bacterium casei* content found in 12 similar cheeses—6 of the raw milk and 6 of the pasteurized milk—

made on successive days. The two cheeses selected were among the best flavored of the lot and are typical cheeses of the raw-milk and pasteurized-milk types. The Cheddar flavor was already developing in the raw-milk cheese when first examined for flavor at 18 days. This flavor increased

TABLE XV.—The correlation of the development of the *Bacterium casei* group and the pungent taste in raw-milk and pasteurized-milk cheese

Age.	Cheese No. 307 C (raw milk).		Cheese No. 307 (pasteurized milk).	
	Number of individuals of <i>Bacterium casei</i> per gram of cheese.	Flavor.	Number of individuals of <i>Bacterium casei</i> per gram of cheese.	Flavor.
Days.				
2	10, 000			
3	10, 000		10, 000	
4	100, 000		10, 000	
5	100, 000		10, 000	
7	10, 000, 000		1, 000, 000	
9	100, 000, 000		1, 000, 000	
11	1, 000, 000		1, 000, 000	
14	10, 000, 000		10, 000, 000	
18	10, 000, 000	Cheddar, developing.	1, 000, 000	Cottage-cheese.
21	10, 000, 000		100, 000	
29	100, 000, 000		10, 000, 000	
36	100, 000, 000		10, 000, 000	
42	10, 000, 000		10, 000, 000	
49	10, 000, 000	Strong Cheddar.		Only a sour taste.
56	10, 000, 000		1, 000, 000	Do.
71	10, 000, 000	Strong Cheddar.	100, 000, 000	Clean, sour taste; no Cheddar.
98	10, 000, 000	Strong Cheddar; getting sharp.	10, 000, 000	Do.
108	10, 000, 000		100, 000, 000	Do.
150	1, 000, 000	Strong Cheddar; sharp.	10, 000, 000	Do.
218	10, 000, 000	Extremely sharp.	1, 000, 000	Acid flavor has disappeared. Mild sharpness.
311		Intensely sharp.		Good cheese. Sharp.

in strength until the ninety-eighth day, when the pungent taste became evident. As the cheese aged, the sharpness became more and more intense. In the pasteurized-milk cheese no other flavor than a sour taste was apparent until the cheese was about 7 months old, when it possessed a mild, sharp taste.

Jensen (1904, p. 356) has shown that the *Bacterium casei* group is active in breaking down the casein of milk to which calcium carbonate is added and maintains that the casein is not peptonized, but is split directly into monoamino acids.

Van Slyke and Hart (1903) have shown that there is a constantly increasing percentage of monoamino acids in ripening Cheddar cheese. At three months more than one-third of the water-soluble nitrogenous compounds is in this form, and later a much larger percentage. The evidence seems to point to the fact that in raw-milk Cheddar cheese and

in the pasteurized-milk cheese the *Bacterium casei* group common to both of these types of cheese is responsible for the biting taste which is characteristic of the well-ripened cheese.

Jensen has explained the action of the *Bacterium casei* group in producing amino acids as due to an "endoerepsin" set free by the dead cells rather than to the activity of the living bacteria, because he found the greatest amount of amino acids formed after the bacteria were for the most part dead (Jensen, 1912).

Whatever may be the condition in the Emmenthaler cheese which Jensen studied, this explanation for the activity of the *Bacterium casei* group in Cheddar cheese is not necessary. It appears to be certain that the living bacteria were active throughout the ripening period in this type of cheese. Cheese No. 17 R (Table IX) and cheese No. 307 C (Table XV) both demonstrated this fact. In cheese No. 17 R there were 10,000,000 living bacteria of the *B. casei* group per gram of cheese when 8 months old; cheese No. 307 C contained a similar number at 7 months. These bacteria did not appear to be living in a latent condition, for all three varieties of *B. casei* readily grew upon an agar made with only an extract of the cheese for a food substance. Every time an analysis was made of cheese No. 17 R such an agar was prepared on the day the analysis was made, using for the cheese agar a part of the plug which served for bacteriological analysis. Thus, the cheese organisms were submitted for development upon the same food substance which served them in the cheese itself.

The result of the bacterial count upon this cheese agar was only slightly smaller than the total count upon the casein agar. Every variety of cheese organisms grew upon this medium. On the eighty-ninth day, when 32,000,000 organisms per gram of cheese developed colonies on the casein agar and of the 10 colonies isolated none belonged to the *Bacterium casei* group, 20,000,000 colonies developed upon the cheese agar, of which 20 per cent, or 4,000,000 bacteria per gram, belonged to the *B. casei* group. The flask dilutions on that same date showed 1,000,000 bacteria of this group per gram of cheese. Thus, it is shown that several millions of living organisms of the *B. casei* group are present in a normal Cheddar cheese 3 months of age, and the cheese itself provides suitable food for development. It is most probable that under these conditions the living bacteria are active in the cheese.

#### INFLUENCE OF BACTERIUM CASEI IN STARTERS FOR PASTEURIZED-MILK CHEESE

Many experimental pasteurized-milk cheeses have been made with pure-culture starters, to determine their influence upon the production of flavor.

In the first series some variety of *Bacterium casei* was added to a number of the cheeses, together with the *B. lactis acidii*. When variety "a" was added, there was a tendency for the cheese to become "acid injured"—strongly acid, friable, and opaque. The use of variety "b" as a starter

was more likely to bring about this condition. When variety "c" was used, the cheese was almost certain to be ruined by the acid before it was a month old. This variety was isolated only once from the 21 normal raw-milk cheeses which have been studied with reference to the varieties; and then there were present less than 500,000 bacteria per gram of cheese. *B. casei*, c, has been isolated, however, from three raw-milk cheeses which had a sour taste rather than the Cheddar flavor. Therefore this variety can not be regarded as a necessary organism in normal ripening. It is likely that it never occurs in large numbers in the young cheese without causing injury. *B. casei*, varieties "a" and "b," are about equally distributed in normal Cheddar cheese, where they usually occur together and perform an active part in the ripening changes. It has been noted that the *B. casei* groups develop gradually in the pasteurized-milk cheese, as they do in the raw-milk cheese, although usually more slowly. The introduction of this group as a starter, however, resulting in abnormally large numbers of *B. casei* in the early ripening period, is detrimental to the cheese.

In subsequent series of experiments with pasteurized-milk cheese this group of organisms was never added to the starter.

#### INFLUENCE OF BACTERIUM LACTIS ACIDI UPON FLAVOR PRODUCTION

When *Bacterium lactis acidi*, a or b, is used for a starter in pure culture in pasteurized-milk cheese, an acid taste is produced which is characteristic of the ordinary pasteurized-milk cheese made with the use of a commercial starter. No suggestion of a Cheddar flavor is ever obtained. If *B. lactis acidi*, d, is added to the milk, there is almost always produced a peculiar flavor, which, as it intensifies with continued ripening, becomes decidedly bitter. Out of 14 experimental cheeses in which 0.75 per cent, the ordinary quantity used for a starter, of a pure culture of this variety was used or in which a mixture of several pure cultures was used with *B. lactis acidi*, d, in large proportions, the unpleasant flavor has developed some time between the fourth and fourteenth weeks. Usually the cheese becomes bitter by the time it is 2 months old. In only one instance has the bitterness failed to develop before the fourteenth week. The figures in Tables VIII and IX show that *B. lactis acidi*, d, was isolated from normal raw-milk cheese with sufficient frequency that it may be concluded that it is always present in this type of cheese in large numbers. It no doubt contributes to the characteristic Cheddar flavor under the conditions obtaining in a normal cheese. But it is certain that a large amount of this variety is not suitable for use in the starter for pasteurized-milk cheese.

#### INFLUENCE OF THE COCCUS GROUPS IN THE PRODUCTION OF FLAVOR

From the frequency of occurrence of all four varieties of the streptococci in normal Cheddar cheese in percentages ranging as high as 50, as shown in Tables VIII and IX, it is most certain that this group is

active in the ripening changes. The by-products of this group of organisms are entirely different from those of the *Bacterium lactis acidii* group. Instead of a large quantity of lactic acid, with small quantities of other acids, as formed by the latter group, the streptococci produce no lactic acid, but produce large amounts of acetic acid, with smaller percentages of propionic, butyric, and caproic acids (Hart, Hastings, Flint, and Evans, 1914). The streptococci were also shown to produce small amounts of ammonia, a by-product not found in *B. lactis acidii* cultures. It is therefore to be expected that the large numbers of streptococci present in Cheddar cheese have a decided influence upon flavor development. The influence of individual cultures of this group in the ripening of pasteurized-milk cheese will be discussed later.

Bacteria of the Micrococcus group were isolated from normal Cheddar cheese in percentages as high as 40. It does not appear that this group of organisms is of primal importance in the production of Cheddar flavor, however, for they have been found commonly in pasteurized-milk cheese in numbers comparable with those found in the raw-milk cheese with well-developed flavor. When added to the cheese in large percentages of the starter, a bitterness is always produced within a few weeks.

#### EXPERIMENTS IN THE PRODUCTION OF DESIRABLE FLAVORS IN PASTEURIZED-MILK CHEESE

The study of the bacterial content of normal Cheddar cheese has demonstrated the fact that the flora is varied and is made up of several varieties of all four groups of cheese organisms. Therefore, in the attempt to prepare starters which might develop Cheddar flavors in the pasteurized-milk cheese it appeared reasonable to mix pure cultures together for the cheese inoculation, choosing the varieties most frequently found in Cheddar cheese with the well-developed flavor. Many experimental cheeses have been made with the use of such starters, the mixtures being made up of from two to nine pure cultures in various combinations and varying percentages, in order to determine which of the varieties in addition to *Bacterium lactis acidii* might improve the acid flavor of the pasteurized-milk cheese.

The difficulties in preparing a starter which will reinstate in pasteurized milk a flora which will simulate raw milk well ripened for cheese making are apparent, even though the relative percentages of each group and variety were better known.

Out of 20 experimental cheeses which were inoculated with *Bacterium lactis acidii*, a or b, together with one of the other varieties of this group or some variety of the coccus groups or with some mixture of these pure cultures, there was usually obtained a better flavor in the young cheese than in the control cheese inoculated with a pure culture of *B. lactis acidii*, a or b, alone. But by the time the cheese was well ripened a

bitterness had usually developed. It was observed that, as in the *B. lactis acidii* group, the coccus cultures with a high ability to ferment the more complex test substances were likely to produce bitterness when inoculated in large percentages. The fact that some of these cultures with high fermenting ability were included in almost all of the mixtures is thought to account for the development of bitterness in this series of experimental cheeses. Nevertheless these organisms may participate in the production of a good Cheddar flavor under the conditions for their development in the raw-milk cheese. In only one cheese of this series was a semblance of a Cheddar flavor obtained. This cheese was inoculated with the following mixture: *B. lactis acidii*, b, 42 per cent; *B. lactis acidii*, d, 48 per cent; *Streptococcus*, c, and *Micrococcus*, b, each 5 per cent. At three months there was an unmistakable resemblance to a Cheddar flavor. At four months the cheese scored as high as 94.6 per cent when examined by experts. Thus, the Cheddar flavor was obtained with this combination of cultures, most of which produced bitterness when used individually.

It was observed that several cheeses of this series, to which there was added a considerable percentage of *Streptococcus*, b, developed a pleasant flavor, an improvement upon the acid flavor of *Bacterium lactis acidii* alone, although it was not the Cheddar flavor. A third lot of experimental pasteurized-milk cheeses was made, in which the value of *Streptococcus*, b, in various proportions, together with *B. lactis acidii*, b, was tested. Three vats of cheese were made on each of three successive days. In series A, 50 per cent or more of the starter was a culture of *Streptococcus*, b; in series B this organism made up  $33\frac{1}{3}$  per cent or less of the starter. In series C the starter was a pure culture of *B. lactis acidii*, b. In Table XVI the scores for the cheeses are given, as determined by expert judges. The average score of the three judges for each cheese is given, 100 being perfect.

TABLE XVI.—The effect upon flavor development of the use of various percentages of *Streptococcus*, b, in the starter for pasteurized-milk cheese

Cheese No.	Series A.		Series B.		Series C.	
	Inoculation.	Score.	Inoculation.	Score.	Inoculation.	Score.
20	<i>Bacterium lactis acidii</i> , b (50 per cent); <i>Streptococcus</i> , b (50 per cent).	92.4	<i>Bacterium lactis acidii</i> , b (66 $\frac{2}{3}$ per cent); <i>Streptococcus</i> , b (33 $\frac{1}{3}$ per cent).	92.7	<i>Bacterium lactis acidii</i> , b (100 per cent).	90.9
21	<i>Bacterium lactis acidii</i> , b (50 per cent); <i>Streptococcus</i> , b (50 per cent).	90.2	do.....	91.5	do.....	91.7
22	<i>Bacterium lactis acidii</i> , b (50 per cent); <i>Streptococcus</i> , b (50 per cent).	94.0	<i>Bacterium lactis acidii</i> , b (75 per cent); <i>Streptococcus</i> , b (25 per cent).	93.8	do.....	91.0
	Average.....	92.2	.....	93.3	.....	91.2



It will be observed that for every day's make the cheese of series B, with the smaller percentages of *Streptococcus*, scored higher than series C, with the pure *Bacterium lactis acidii* starter. Series A, with the larger percentages of *Streptococcus*, scored higher than series C in two out of the three cheeses. Series B averaged 2.1 points higher than the series C, and series A averaged 1 point higher than series C.

The differences in flavor, however, were greater than the figures indicate, since the scores were made upon the commercial value of the cheese. The cheeses of series C had the acid flavor typical of a young pasteurized-milk cheese made with the ordinary commercial starter. The cheeses of series B had none of this acid flavor, but instead they possessed a mild flavor which was more agreeable to the taste of a number of persons knowing nothing of the experiment, to whose judgment the samples were submitted. All were agreed that the cheeses of series B lacked the acid taste. The cheeses of series A also differed greatly from those of the series C. They did not have the pleasant flavor of series B, however, but had an acid flavor essentially different from that of series C and inferior to the mild flavor of series B.

These experiments with the use of the *Streptococcus*, b, culture in starters demonstrate the fact that the addition of comparatively small percentages of this kind of culture brings about a decided difference in flavor, which is regarded as an improvement by all who have passed judgment in the matter. The larger percentages of streptococci give less desirable flavors than the smaller percentages. It seems probable that further experimentation with this and other cultures of *Streptococcus*, b, in combination with *Bacterium lactis acidii* may give results which will be of practical value in the improvement of flavors in pasteurized-milk cheese. And it does not seem unreasonable to hope that further experimentation with the *B. lactis acidii*, a or b, together with smaller percentages of *Streptococcus*, b, as the basis for a starter and with various combinations of very small percentage of the other cheese organisms, Cheddar flavors may be obtained in the pasteurized-milk cheese.

#### SUMMARY

- (1) The organisms constantly found in Cheddar cheese in such numbers as to indicate they must function in the ripening process are included in four groups: First, the *Bacterium lactis acidii*; second, the *B. casei*; third, *Streptococcus*; fourth, *Micrococcus*.
- (2) On the basis of the fermentation powers each of the four groups may be divided into a number of varieties.
- (3) The distribution of the varieties of the four groups in Cheddar cheese prepared from raw milk has been studied, as has also been done with cheese prepared from pasteurized milk.

(4) The flora of raw-milk cheese is varied and consists of all the varieties into which the four groups were divided.

(5) The flora of pasteurized-milk cheese, with the exception of the *Bacterium casei* group, is dependent upon the flora of the starter.

(6) The *Bacterium casei* group is apparently responsible for the pungent taste that develops late in the ripening period of both raw-milk and pasteurized-milk cheeses. It is probable that growth of this group continues during the major part of the ripening period.

(7) The action of two or more organisms growing together is not the sum of their individual actions when growing alone. When growing together, they may attack substances that neither can attack alone, or they may produce a larger quantity of acid than the sum of the quantities that either can produce alone.

(8) When added to pasteurized milk, the organisms of the *Bacterium casei* group produce a sour taste in the cheese during the early part of the ripening period.

(9) No Cheddar flavor is obtained in pasteurized-milk cheese when the organisms of the *Bacterium lactis acidi* group alone are used as starters. The varieties that are able to ferment the more complex substances are likely to produce a bitter taste.

(10) Starters composed of both *Bacterium lactis acidi*, b, and Streptococcus, b, when added to pasteurized milk, improve the quality of the cheese. It does not seem unreasonable to hope that starters may be obtained that will give the characteristic Cheddar flavor to the cheese prepared from pasteurized milk.

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## RELATION OF THE ACTION OF CERTAIN BACTERIA TO THE RIPENING OF CHEESE OF THE CHEDDAR TYPE<sup>1</sup>

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### INTRODUCTION

The ripening of Cheddar as well as other varieties of cheese has been studied by a large number of investigators. The decomposition of the protein and the nitrogenous substances thereby produced have been quite thoroughly studied in Europe and America. These studies have involved both hard and soft cheeses. The nature of the nonnitrogenous substances formed during fermentation in cheese, such as fatty acids, alcohol, esters, and aldehydes, has received less attention, but there can be no doubt that they contribute to the aroma and also to the taste of the product. In their relation to flavor they are equally, if not more, important than the nitrogenous substances.

According to present views, the factors involved in the curing of Cheddar cheese are the pepsin contained in the rennet; the activating lactic acid formed from lactose fermentation; galactase, the proteolytic enzyme of milk; other inherent enzymes of milk; and certain biological agents other than those simply concerned in the first lactose fermentation.

Investigations at the Wisconsin Agricultural Experiment Station and the New York (Geneva) Agricultural Experiment Station have shown that the inherent enzymes of milk and rennet fail to produce the typical Cheddar cheese flavor. This has led to a more extensive investigation of the biological factors of Cheddar cheese ripening.

In an earlier publication from the Wisconsin station (Suzuki, Hastings, and Hart, 1910)<sup>2</sup> both volatile acids and esters were separated and identified from curing Cheddar cheese, but no data concerning the factors operative in their origin were presented. In a later publication (Hastings, Evans, and Hart, 1912) work was reported that showed the presence and persistence in this type of cheese of three groups of organisms, the *Bacterium lactis acidii* group, the *B. casei* group,<sup>3</sup> and possibly a group of coccus forms.

<sup>1</sup> Work of the Department of Agriculture in cooperation with Wisconsin Agricultural Experiment Station.

<sup>2</sup> Bibliographic citations in parentheses refer to "Literature cited," p. 214-215.

<sup>3</sup> The organisms of the *Bacterium casei* group appear in the literature under a number of names, the most common being "lactic bacilli," "*Bacterium bulgaricus*" or "*Bacterium bulgaricum*," "*Bacterium casei*," and the "yoghurt bacillus." The name "*Bacterium casei*" will be used in this article.

In a preliminary investigation of the nonnitrogenous constituents of Cheddar cheese (unpublished data) the very pronounced differences that were expected in the quantity and variety of volatile acids, esters, and alcohols in good and poor types of cheese were not found. But since there were certain differences which could be only of biological origin, it was believed essential to this problem that the substances formed by the specific groups of organisms normally present in cheese be more carefully studied. For this reason it was decided to extend the investigation to an examination of the substances produced by representatives of the groups that had been found to be present in cheese in such numbers that it was evident that they must be of importance in the ripening process. In this way it was hoped to find the groups of organisms to which might be assigned responsibility for the production of definite nonnitrogenous compounds that could be correlated with flavor production. The compounds particularly sought were the alcohols and esters and caproic and butyric acids. Formic, acetic, propionic, lactic, and succinic acids were also included in the list of substances to be isolated. To some extent the sources of these bodies were also studied. This paper is a progress report on this phase of our work.

Ferdinand Cohn (1875) was the first to connect the cheese-ripening process with the activity of bacteria. Duclaux (1894, p. 265-267) considered that the volatile fatty acids found in cheese arose from the action of the bacteria on casein and from the hydrolysis of the fat. He believed also that butyric acid was a source of other volatile acids, the butyric acid arising partly from fat decomposition and partly from decomposition of casein. Baier (1895), Von Klecki (1896), and Weigmann (1896, 1898) believed butyric-acid bacteria to be of importance in the ripening of cheese. Von Freudenreich (1897, 1902) attributed to the lactic-acid bacteria the principal rôle in the ripening process, especially in Emmenthaler cheese. Jensen (1904) in his work on Emmenthaler and other European cheeses has contributed much to the general subject of the chemistry and bacteriology of cheese ripening and in agreement with Von Freudenreich gives to the lactic-acid-producing organisms very great importance in the ripening process. Suzuki, Hastings, and Hart (1910) have investigated the source of the volatile acids and the forms of lactic acid found in American Cheddar cheese, studying in connection with these subjects the decomposition of lactose, lactates, fat, proteins, and glycerin.

The constituents of a fresh cheese mass which can be sources of the nonnitrogenous bodies under consideration are paracasein, fat, lactose, lactates, and citrates. From paracasein there arises gradually during the ripening process a series of nitrogenous compounds which have been fairly well investigated (Winterstein; Steinegger; Benecke and Schulze; Van Slyke and Hart, Apr., 1903, and July, 1903; Dox). At least three of these—namely, cadaverin, putrescin, and ammonia—are slightly

volatile and probably can influence the aroma of cheese. The other nitrogenous end-products undoubtedly are factors in the flavor production, and influence taste.

It is known that proteolysis gives rise also to volatile fatty acids, particularly butyric acid. In addition, milk fat, which is present to a large extent in the cheese, is a source of caproic and butyric acids through bacterial and enzymic action. The glycerin of the fat after hydrolysis by biological agencies is a source of acetic and propionic acids under the influence of further fermentation (Suzuki, Hastings, and Hart, 1910). That decomposition of fat occurs during cheese ripening, giving rise to caproic and butyric acids, has been shown by a number of workers. Duclaux (1894, p. 286) found that this occurred to quite an extent, giving rise to free volatile fatty acids. Weigmann and Backe (1898) point to the presence in ripe cheese of free nonvolatile acids, such as oleic, palmitic, and stearic, as an indication of fat decomposition in the cheese-ripening process. Kirsten (1898, p. 1), however, thought these higher acids could arise from paracasein and claimed that fat decomposition in ripening cheese is almost imperceptible. Jensen (1904, p. 319) has shown that very probably fat decomposition does take place with production of fatty acids during cheese ripening. The lactose fermentation produces, besides lactic acid, formic and propionic acids, and under certain conditions butyric and caproic acids also are formed (Suzuki, Hastings, and Hart, 1910). Calcium lactate, according to Fitz (1878, p. 51; 1879, p. 479; 1880, p. 1309; 1881, p. 1084), is a source of acetic and propionic acids, and under certain conditions also of caproic and valeric acids. Jensen (Von Freudenreich and Jensen, 1906, p. 320) and Troili-Petersson (1909, p. 333) have shown that the lactates in Emmentaler cheese are fermented by organisms with the production of propionic and acetic acids and  $\text{CO}_2$ . Troili-Petersson has also shown that glycerin may be a source of propionic acid.

In an extended investigation (Evans, Hastings, and Hart, 1914) of the flora of American Cheddar cheese it has been shown that the organisms fall into four groups, the *Bacterium lactis acidii*, the *B. casei*, and two coccus groups.

The substances produced by the coccus groups form the principal theme of this paper. In addition, data are given on the substances formed from two representatives of the *Bacterium casei* group. In the following work pure cultures of several of the coccus forms known to occur in American Cheddar cheese were inoculated into flasks containing 300 c. c. of sterile separated milk and kept at a temperature of 35° C. for at least two months before being examined. No alkali whatever was added to the milk. The high-acid-producing organisms (*B. casei* group) were also inoculated into flasks of milk similarly prepared and incubated. Each culture was put up in duplicate flasks. The methods of analysis used were those described by Suzuki, Hastings, and Hart (1910). All

flasks subjected to analysis were examined to ascertain their freedom from growth of other organisms.

It has been observed that active lactic acid is the main form of this acid in fresh cheese curd, but that it rapidly changes to the racemic variety. In addition to the foregoing studies on substances formed by bacteria, this paper also includes some work done on the agencies which cause these changes in the form of lactic acid present in cheese and which take place during the earlier period of cheese ripening.

In the preceding article (Evans, Hastings, and Hart) the presence of coccus forms in normal Cheddar cheese is demonstrated. It is shown that nonliquefying cocci which ferment lactose in milk cultures are always present, in percentage of the total bacterial content ranging upward to 50. The cocci are distinguished from the *Bacterium lactis acidi* group by their morphology and by the extent of reduction of litmus in milk cultures. In cultures of the *B. lactis acidi* group the cells are in pairs, and some or all of the cells are elongated; there is always a characteristic reduction of litmus. The cocci include those cultures in which the cells are spherical. The complete reduction of litmus beneath the surface layer, characteristic of the *B. lactis acidi* group, does not take place.

A classification of the cocci occurring in this type of cheese is made. They are divided into two groups on the basis of morphology: Streptococci and micrococci. Those occurring in pairs are included with the streptococci, together with those which form chains of varying lengths. The micrococci are the Coccaceæ which divide in two planes; consequently the cells appear in pairs, fours, or bunches. Most of the cultures of this group produce a heavy growth upon agar slant, which is often of some shade of yellow. A further differentiation of the groups into varieties is made on the basis of fermentation of the following test substances: Lactose, salicin, sucrose, glycerin, and mannit. This classification of the cocci is given in Table I. The substances produced by representatives of several of these varieties have been analyzed, and the data are presented in Tables II to X.

TABLE I.—Differentiation of the coccus groups into varieties

Group.	Variety.	Production of acid in—				
		Lactose.	Salicin.	Sucrose.	Glycerin.	Mannit.
Streptococcus.....	a	—	—	—	—	—
	b	+	—	+	+	—
	c	+	+	+	+	+
	d	+	+	+	+	+
Micrococcus.....	b	+	+	—	—	—
	c	+	+	+	—	—
	d	+	+	—	—	+

## ANALYSIS OF THE DECOMPOSITION PRODUCTS OF STERILE MILK

In Table II are given the quantities of the various substances found in 300 c. c. of the sterile milk after incubation for four months.

TABLE II.—*Decomposition products found in 300 c. c. of sterile milk*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity.		
	Flask 1.	Flask 2.	Average.
Total volatile acids.....	11.763	12.567	12.164
Formic acid.....	5.748	5.348	5.548
Acetic acid.....	5.566	6.756	6.160
Propionic acid.....	.000	.000	.000
Butyric acid.....	.000	.000	.000
Caproic acid.....	.440	.403	.456
Acids from alcohols.....	.954	.050	.802
Acids from esters.....	.685	.600	.640
Succinic acid.....	.000	.000	.000
Total lactic acid.....	.000	.000	.000
Racemic lactic acid.....			
Active lactic acid.....			

The occurrence of formic and acetic acid in the controls may be due to the decomposition of lactose in the process of sterilization. Formic acid, at least, has been observed in milk heated for some time at high temperature (Cazeneuve and Haddon).

The cultures of *Streptococcus b<sub>1</sub>* (Table III) were 8 weeks old when analyzed. Little digestion of the medium was apparent. The medium had a clean, sweet, fruity, or nutty taste and odor, was gray white in color, and somewhat slimy. This organism was present in the cheese to the extent of 10,000,000,000 per gram when isolated. The cheese was 77 days old when examined. It had a very mild Cheddar flavor, which developed late in the curing, and it afterwards developed a good sharp flavor.

TABLE III.—*Substances formed by the action of Streptococcus b<sub>1</sub>*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids.....	52.689	54.030	12.164	40.525	42.766
Formic acid.....	.000	.000	5.548	—5.548	—5.548
Acetic acid.....	49.920	50.810	6.160	43.760	44.050
Propionic acid.....	2.575	4.120	.000	2.575	4.120
Butyric acid.....	.000	.000	.000		
Caproic acid.....	.104	.000	.456		
Acids from alcohols.....	.769	.621	.802		
Acids from esters.....	.850	.840	.640	.210	.200
Succinic acid.....	.000	.000	.000		
Total lactic acid.....	Trace.	.000	.000		
Racemic lactic acid.....					
Active lactic acid.....					



*Streptococcus b<sub>1</sub>* decomposed all the formic acid present in the milk and produced large quantities of acetic and a little propionic acid. Esters were produced in small amounts. No lactic acid was found.

The cultures of *Streptococcus b<sub>2</sub>* (Table IV) were 11 weeks old when analyzed. In both flasks a soft curd was deposited. The contents of flask 1 had a sharp nutty odor and flavor. Flask 2 had a sharp, acid, unpleasant taste and a sharp, rancid smell suggesting butyric acid. The cheese from which the isolation was made was 101 days old when examined. It contained this organism in numbers of 1,000,000,000 per gram. The cheese possessed a good Cheddar flavor when 2 weeks old. Later, a sharpness developed, but the cheese remained good for 6 months.

TABLE IV.—Substances formed by the action of *Streptococcus b<sub>2</sub>*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids.....	49.138	55.152	12.165	36.973	42.987
Formic acid.....	3.640	6.333	5.548	—1.908	.789
Acetic acid.....	38.370	41.059	6.160	32.210	34.890
Propionic acid.....	4.903	5.138	.000	4.903	5.138
Butyric acid.....	.558	.563	.000	.558	.563
Caproic acid.....	1.667	2.059	.456	1.211	1.603
Acids from alcohols.....	8.607	3.552	.802	7.805	2.750
Acetic acid.....	8.209	3.187	.....	7.497	2.385
Propionic acid.....	.398	.365	.....	.398	.365
Acids from esters.....	.250	1.030	.640	.....	1.290
Total lactic acid.....	.000	.000	.000	.000	.000
Racemic lactic acid.....	.....	.....	.....	.....	.....
Active lactic acid.....	.....	.....	.....	.....	.....

This form of coccus decomposed a part of the formic acid originally present in the medium. The increase in acidity was mainly due to acetic acid, but some propionic and a little caproic acid were also formed. The interesting point in connection with this organism, however, was the strong production of alcohols, amounting to a quantity equivalent to nearly 8 cubic centimeters of decinormal acid. Most of this alcohol was ethyl, a little propyl alcohol making up the remainder. In one flask a marked production of esters was also noted. No lactic acid was produced.

The cultures of *Streptococcus b<sub>2</sub>* (Table V) were 4½ months old when analyzed. Flask 1 had a yellowish colored solution over a firmly deposited custard-like curd. The solution was acid to litmus and had a pleasant, slightly acid smell. The residue in flask 2 was less than that in flask 1 and was covered by a brown-colored solution which was acid to litmus. Its odor was similar to that of flask 1, but was more pronounced, giving a suggestion of cheese odor.

TABLE V.—Substances formed by the action of *Streptococcus b*<sub>1</sub>

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids.....	88.799	90.474	14.530	74.269	75.944
Formic acid.....	3.153	.000	8.295	-5.142	-8.295
Acetic acid.....	68.190	69.150	6.135	62.055	63.015
Propionic acid.....	10.209	9.970	.000	10.209	9.970
Butyric acid.....	2.090	3.821	.000	2.090	3.821
Caproic acid.....	5.157	7.533	.100	5.057	7.433
Acids from alcohol.....	7.743	5.158	.375	7.368	4.783
Formic acid.....	.313	.000	.313	.....	.....
Acetic acid.....	6.908	4.612	.....	6.533	.....
Propionic acid.....	.522	.540	.....	.522	.546
Acids from esters.....	4.739	4.856	.833	3.906	4.023
Formic acid.....	.443	.000	.....	.443	.000
Acetic acid.....	3.979	4.051	.....	3.146	3.818
Propionic acid.....	.317	.205	.....	.317	.205
Citric acid.....	65.982	47.090	84.800	-18.818	-37.710
Total lactic acid.....	.000	.000	.000	.000	.000
Racemic lactic acid.....	.....	.....	.....	.....	.....
Active lactic acid.....	.....	.....	.....	.....	.....
Ammonia..... grams.....	.045	.052	.023	.022	.029

The data in Table V show the same general indications as the data in Table IV. A larger increase was shown in total volatile acidity. Comparatively a much greater increase was noted in the case of butyric and caproic acids. A greater increase was also evident in alcohol and ester production, ethyl alcohol and acetic acid in ester combination predominating. A minute quantity of formic acid previously existing in ester compounds and also from methyl alcohol was recovered. Since probably only about 6.2 per cent of esters are recovered in the method used, the amount of esters actually found indicates a preexisting quantity of those bodies equivalent to 64.8 c. c. N/10. This quantity is greater than the ester content of any cheese examined. If this organism is an agent which produced esters in cheese, as the data indicate, it would, however, be subjected to inhibiting influences in the cheese mass and probably not be able to form esters in such great quantities as when in pure culture.

The culture of *Streptococcus d*<sub>1</sub> (Table VI) was 2½ months old when analyzed. The medium had a pleasant nutty odor and a slightly acid taste. A soft custard-like curd had formed; that in flask 2 showed the greater bacterial action, having a more acid odor, but the taste was similar to that in flask 1. The curds were alike, with a clear supernatant liquid. The cheese from which this organism was isolated was 133 days old and contained the organism to the extent of 170,000,000 per gram. It had a mild Cheddar flavor after 4 months of curing.

TABLE VI.—Substances formed by the action of *Streptococcus d.*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids.....	19.142	Lost.	14.530	4.612	Lost.
Formic acid.....	.000		8.295	—8.295	
Acetic acid.....	18.310		6.135	12.175	
Propionic acid.....	.692		.000	.692	
Butyric acid.....	.000		.000		
Caproic acid.....	.140		.100		
Acids from alcohol.....	.680	1.000	.375		0.625
Acids from esters.....	.915	.725	.833	.082	
Citric acid.....			84.800		
Total lactic acid.....		Trace.	.000		
Racemic lactic acid.....					
Active lactic acid.....					

All the formic acid was destroyed by this coccus, and but a comparatively small quantity of acetic acid formed. The activity of this organism was apparently slight; but slight traces of esters were found in one flask and no lactic acid in either flask. The apparent contradiction that there exists a larger content of acetic acid than total volatile acids is due to the destruction of formic acid.

The cultures of *Streptococcus d.* (Table VII) were 2 months old when analyzed. They possessed a pleasant nutty taste and smell. No digestion was apparent. The contents of flask 2 had a trifle more pronounced flavor and odor than in flask 1, but were of the same quality. The cheese from which the organism was isolated was 75 days old and contained this coccus to the extent of 10,000,000,000 per gram. No typical Cheddar flavor had developed. After 5 months the cheese developed a sharper taste, but still was fairly good.

TABLE VII.—Substances formed by the action of *Streptococcus d.*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids.....	58.313	66.664	14.530	43.783	52.134
Formic acid.....	.780	1.029	8.295	—7.506	—7.266
Acetic acid.....	53.140	59.530	6.135	47.005	53.395
Propionic acid.....	4.029	5.700	.000	4.020	5.700
Butyric acid.....	.000	.301	.000		
Caproic acid.....	.355	.104	.100		
Acid from alcohols.....	.800	.765	.375	.425	.390
Acid from esters.....	.770	.540	.833		
Citric acid.....	7.100	7.100	84.800	—77.700	—77.700
Total lactic acid.....	.000	.000	.000		
Racemic lactic acid.....					
Active lactic acid.....					

This culture produced acetic acid almost entirely. As practically all of the citric acid had been destroyed, it may be assumed that this acid was in part the source of the acetic acid. That citric acid can be broken down by certain organisms has already been pointed out by Bosworth and Prucha (1910).

The cultures of *Micrococcus b* (Table VIII) were  $3\frac{1}{4}$  months old when analyzed. The flasks were alike in appearance and odor. The cheese was 43 days old and had a mild Cheddar flavor when the isolation was made. This organism was present in the cheese to the extent of 1,600,000 per gram.

TABLE VIII.—Substances formed by the action of *Micrococcus b*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids.....	72.785	67.529	12.164	60.621	55.365
Formic acid.....	4.857	3.667	5.548	—	621
Acetic acid.....	64.305	59.740	6.160	58.145	53.580
Propionic acid.....	3.220	3.914	.000	3.220	3.914
Butyric acid.....	.000	.000	.000	.000	.000
Caproic acid.....	.403	.208	.456	.....	.....
Acids from alcohol.....	.409	Lost.	.802	.....	.....
Acids from esters.....	.500	.405	.640	.....	.....
Succinic acid.....	.000	.000	.000	.000	.000
Total lactic acid.....	17.152	.000	.000	17.152	.000
Racemic lactic acid.....	3.730	.000	.000	3.730	.000
Active lactic acid.....	13.422	.000	.000	13.422	.000

Acetic acid shows the only large increase among the volatile acids. In flask 1 a small quantity of lactic acid had developed. Most of it was of the active variety.

The cultures of *Micrococcus d* (Table IX) were  $1\frac{3}{4}$  months old when analyzed. Flask 2 showed from its appearance and odor further decomposition and probably more rapid growth of the organism than flask 1.

TABLE IX.—Substances formed by the action of *Micrococcus d*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids.....	29.482	26.614	12.164	17.318	14.450
Formic acid.....	2.484	1.316	5.548	—	3.064
Acetic acid.....	8.370	13.295	6.160	2.210	7.135
Propionic acid.....	4.540	4.988	.000	4.540	4.988
Butyric acid.....	7.811	2.456	.000	7.811	2.456
Caproic acid.....	6.277	4.559	.456	5.821	4.103
Acids from alcohol.....	1.499	1.754	.802	.697	.950
Acids from esters.....	.350	.450	.640	.....	.....
Succinic acid.....	.830	3.300	.000	.830	3.300
Total lactic acid.....	3.396	20.856	.000	3.396	20.856
Racemic lactic acid.....	.000	11.940	.000	.000	11.940
Active lactic acid.....	3.396	8.916	.000	3.396	8.916

It will be noticed that formic acid has decreased. This will be found true for all the organisms studied, the acid probably being decomposed by the organisms themselves. In flask 1 the greatest increase is shown in the butyric-acid content. In flask 2, where greater decomposition and probably more rapid growth of the organisms occurred, the butyric acid is very much less, while the acetic acid has increased. This would indicate a decomposition of butyric acid to a lower acid, as Duclaux suggests in his theory of the formation of acids lower than butyric. All of the volatile acids, except formic, show an increase. A very small quantity of succinic acid was formed, but no esters and very little alcohol were produced. In flask 2 there was quite an amount of lactic acid, of which most was racemic.

The substances formed by a third *Micrococcus* are given in Table X. This culture was not classified as to variety. It was isolated from a cheese when the latter was 44 days old and when the organism was present in numbers amounting to 100,000,000 per gram. After 5 months this cheese developed a good Cheddar flavor. In both flasks the media were light brown in color and had a pleasant nutty odor and taste; they showed slight digestion and were but slightly acid to litmus.

TABLE X.—Substances formed by the action of an unidentified *Micrococcus*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids.....	46.058	52.669	14.530	31.538	38.139
Formic acid.....	.000	.000	8.295	-8.295	-8.295
Acetic acid.....	41.850	37.060	6.135	35.715	30.925
Propionic acid.....	3.999	14.886	.000	3.999	14.886
Butyric acid.....	.138	.289	.000	.138	.289
Caproic acid.....	.081	.434	.100	.....	.....
Acids from alcohols.....	.990	.700	.375	.525	.325
Acids from esters.....	1.000	.540	.833	.167	.....
Citric acid.....	80.900	59.240	84.800	.....	-25.560
Total lactic acid.....	.000	.000	.000	.000	.000
Racemic lactic acid.....	.....	.....	.....	.....	.....
Active lactic acid.....	.....	.....	.....	.....	.....

This organism produced quite a quantity of acetic acid and more propionic acid than any other organism examined. No lactic acid was found.

In order to determine the influence of the presence of alkali on the character of the products formed, a flask of milk to which was added calcium carbonate was inoculated with one of the micrococci. The substances formed were acetic, propionic, butyric, and caproic acids, but no formic acid. The proportion of these acids was very similar to that of the acid formed by *Micrococcus d* and would indicate that the alkali exerted no influence on the character of the substances formed.

From a summary of all the foregoing data it appears that the coccus forms do not produce formic acid, and, with the exception of *Micrococcus* b and d, do not produce lactic acid. In the case of these two strains the form of acid produced was both active and racemic. With the exception of *Micrococcus* d all produce relatively large amounts of acetic acid. *Streptococcus* b<sub>1</sub> produced a fairly large quantity of butyric and caproic acids.

# SUBSTANCES FORMED BY ORGANISMS OF THE BACTERIUM CASEI GROUP

In Tables XI and XII are given data showing the substances formed by the action of a high-acid-producing organism, one of the *Bacterium casei* group. Duplicate flasks 58<sub>1</sub> and 58<sub>2</sub> were prepared from two strains of the same culture obtained from different colonies on an agar plate. The milk media at 7½ months old were light yellow in color, and the curd had settled in a firm mass. Both flasks of 58<sub>1</sub> and flask 1 of 58<sub>2</sub> had a very faint acid odor. Flask 2 of 58<sub>2</sub> had a ripened-cream odor.

TABLE XI.—Substances formed by the action of culture 58<sub>1</sub>

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids.....	35.128	38.063	14.530	20.598	23.533
Formic acid.....	.000	1.387	6.295	-6.295	-4.908
Acetic acid.....	33.420	35.760	6.138	27.285	29.625
Propionic acid.....	1.708	.916	.000	1.708	0.916
Butyric acid.....	.000	.000	.000	.....	.....
Caproic acid.....	.000	.000	.100	.....	.....
Acids from alcohol.....	.250	Lost.	.375	.....	.....
Acids from esters.....	.800	Lost.	.833	.....	.....
Citric acid.....	.000	.000	84.800	-84.800	-84.800
Total lactic acid.....	92.648	99.100	.000	92.648	99.100
Racemic lactic acid.....	44.552	87.800	.....	44.552	87.800
Active lactic acid.....	48.096	11.300	.....	48.096	11.300

TABLE XII.—Substances formed by the action of culture 58<sub>2</sub>

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids.....	41.675	40.928	14.530	27.145	26.498
Formic acid.....	3.408	.984	8.295	-4.887	-7.311
Acetic acid.....	36.130	31.090	6.135	29.995	25.855
Propionic acid.....	2.137	7.954	.000	2.137	7.954
Butyric acid.....	.000	.000	.000	.....	.....
Caproic acid.....	.000	.000	.100	.....	.....
Acids from alcohol.....	.500	.950	.375	.....	.....
Acids from esters.....	1.500	1.000	.833	.667	.167
Citric acid.....	.000	.000	84.800	-84.800	-84.800
Total lactic acid.....	48.540	39.970	.000	38.540	39.970
Racemic lactic acid.....	41.100	26.170	.....	41.100	26.170
Active lactic acid.....	7.440	19.800	.....	7.440	19.800

This organism, as might be expected, shows a marked difference from the coccus group in the character of the substances formed. A large amount of lactic acid, including both the racemic and the active forms, was produced. All the citric acid of the milk was destroyed. Like the coccus forms, this organism also produced much acetic acid, but no formic, butyric, or caproic acid. Culture 58, produced some esters.

#### ESTER FORMATION IN CHEDDAR CHEESE

It has been determined that esters do not appear in Cheddar cheese until it is about 5 weeks old. *Streptococcus b.* (see Table V) produced an ester content in the medium equivalent to 64.8 c. c. N/10. To throw some light on the question whether esters could be formed in the cheese or medium from mere mass action of free alcohol and acid, a trial was made with a mixture of these two substances. It is known that the contact of acetic acid and ethyl alcohol can produce esters even without adding a dehydrating agent. Dilute solutions of pure acid and pure alcohol were mixed and allowed to stand for a few months, and then a very slight excess of KOH solution was added. The alcohol and esters were next distilled off. The distillate was saponified with KOH, acidified with  $H_2SO_4$ , and distilled repeatedly to obtain the acids which had entered into the ester combination. Blank determinations were carried out to check the purity of all chemicals used. The results are given in Table XIII.

TABLE XIII.—Production of ester from the contact of acid and alcohol

Ethyl alcohol.	Acetic acid.	Result.
<i>Per cent.</i>	<i>Per cent.</i>	
1	1	No ester detected.
2	2	Small amount of ester.
5	5	Esters formed.
8	8	Do.
14	14	Do.

Table XIII shows that free acetic acid and alcohol can not form esters in dilute aqueous solutions. Comparing this concentration with that found in cheese, it is probable that the solution of alcohol in the cheese moisture is very dilute—much less than 1 per cent. The greater part of the acids is also combined with basic substances. If these assumptions are accepted, then it can be said that the esters in cheese are probably not produced by mere contact of alcohol and acid but by the intervention of biological activities.

Of course, the question of actual concentration of alcohol or acid in any phase in the cheese mass is not possible of definite statement. There may be very little "free" water in the cheese, most of it being in com-

bination with the cheese colloids; consequently the concentration of acid or alcohol in such water may be very large, thereby affording an opportunity for ester formation by mass action. On the other hand, it must not be assumed that the alcohols or acids are "free" in such a complex system, but may also be in combination with the colloids of the cheese mass. If this last alternative is permissible—and the writers believe it to be true, especially for the acids—then there is some reason, at least, for the assumption that ester formation is not the result of mere contact of acid and alcohol, but occurs through the intervention of some agent which shifts the point of equilibrium in the system toward ester stability.

To determine whether inherent milk enzymes acting in curing cheese could produce esters, alcohols, or volatile acids, a cheese was made from chloroformed milk and kept in an atmosphere of chloroform for 5 months. To determine the volatile bodies, 800 grams of the cheese were submitted to steam distillation, after acidifying with  $H_2SO_4$ . The entire analytical process was conducted as has been described. The results were negative, there being neither acids nor esters. This shows that inherent milk enzymes are not the cause of the production of volatile fatty acids and esters in curing cheese. From this experiment it is apparent that the inherent lipase in milk is either retarded in its action by chloroform or else is very slow in its action.

#### AMMONIA PRODUCTION IN MILK

The origin of ammonia in ripening cheese had been ascribed by Babcock and Russell (1897, p. 161) and Babcock, Russell, Vivian, and Hastings (1899, p. 157) to the action of galactase. In further work on this problem Van Slyke and Hart (1903) showed that in chloroformed cheese, where galactase and pepsin would be the only proteolytic agents present, no ammonia was formed. To throw further light on this problem, cultures of a few of the organisms known to be active in Cheddar cheese were examined for ammonia production. Milk was the medium used for these determinations. A part of this medium, in the case of the coccus group, was distilled directly with  $MgO$ . In the case of *Bacterium casei*, the medium was first treated with tannic acid and salt solution according to the standard methods for separation of the tannin precipitate, and the ammonia determined in the filtrate by distillation with  $MgO$ . In Table XIV are recorded the results.



TABLE XIV.—Quantity of ammonia produced in 300 c. c. of milk by different organisms

Organism.	Quantity of NH <sub>3</sub> found.	Quantity of NH <sub>3</sub> in control.	Quantity of NH <sub>3</sub> formed by organisms.
	Grams.	Grams.	Grams.
Streptococcus b <sub>2</sub> .....	0.0220	0.023	.....
	.0200	.023	.....
Streptococcus b <sub>3</sub> .....	.0450	.023	0.0220
	.0520	.023	.0290
Micrococcus.....	.0690	.023	.0460
Bacterium casei.....	.0427	.026	.0167
	.0382	.026	.0122
Bacterium casei <sub>2</sub> .....	.0367	.026	.0107
	.0453	.026	.0193

No large quantity of ammonia was formed by any of the organisms examined. The difference in the amount of ammonia produced by *Streptococcus b<sub>2</sub>* and *Streptococcus b<sub>3</sub>*—two strains of the same variety—may be due to the fact that *Streptococcus b<sub>3</sub>* grew for more than twice as long a time as did the other. It is clear from Table XIV that some of the biological agencies active in the cheese are capable of forming both acids and ammonia.

#### KINDS OF LACTIC ACID IN CHEESE<sup>1</sup>

In considering lactic acid and its changes in cheese, it will be remembered that lactose disappears from the cheese mass after a very few days of curing; subsequently the lactic acid increases up to the five weeks' stage. At later periods the lactic acid content fluctuates, probably the result of production and decomposition by active organisms. Thus, there seems to be a source of lactic acid other than lactose. A solution of alanin, one of the amino acids arising from casein proteolysis and very closely related to lactic acid, was inoculated with a piece of old cheese, in order to ascertain whether alanin could be a source of lactic acid (Suzuki, Hastings, and Hart, 1910). The results were negative, but it is possible that either the nature of the solution or the age of the cheese was responsible for this result. Additional work on this point is necessary.

It is known that cheese contains lactic acid, which usually is racemic in variety. It has been shown in the preceding article that Cheddar cheese 4 or 5 days old contains both racemic and active lactic acid, the latter being present in much greater amount than the former. The active form gradually decreases until it disappears, while the racemic acid increases and remains. It was found by Salkowski (1909, p. 237) that the transformation of dextro lactic acid into racemic acid on pro-

<sup>1</sup> The work reported in the remainder of this paper was completed before the classification of cheese organisms referred to on page 195 and treated in detail in the preceding article entitled "Bacteria concerned in the production of the characteristic flavor of cheese of the Cheddar type" was adopted; consequently that classification is ignored in the following pages.

longed standing takes place in a meat extract such as Liebig's. In the curing of cheese the disappearance of active lactic acid, as well as the production of racemic lactic acid, takes place rapidly. The early stages of these phenomena were next investigated.

Whey drawn from the vat during the process of cheese making and subjected to analysis for lactic acid gave the results shown in Table XV:

TABLE XV.—Analysis of whey, showing quantity of lactic acid as zinc lactate

Fraction No.	Crystals of zinc lactate.	Water of crystallization. <sup>1</sup>
	Grams.	Per cent.
1.....	2.4587	13.07
2.....	.7902	12.27

<sup>1</sup> The theoretical percentage for water of crystallization in active zinc lactate is 12.89.

Fresh curd from which the above whey was drawn was kept at 35° C. for 3 days and gave the following results (Table XVI):

TABLE XVI.—Analysis of fresh curd, 3 days old, showing quantity of lactic acid as zinc lactate

Fraction No.	Crystals of zinc lactate.	Water of crystallization. <sup>1</sup>
	Grams.	Per cent.
1.....	2.0837	17.99
2.....	.1052	17.43

<sup>1</sup> The theoretical percentage for water of crystallization in racemic zinc lactate is 18.18.

It is seen that whey contained active lactic acid, while curd or cheese only 3 days old and kept at 35° C. contained nearly all its lactic acid in the racemic form. It is probable that a second group of organisms follows the early action of the predominating active lactic-acid producers in the cheese during the first 3 days. There is also a possibility that a somewhat different sequence of bacterial life occurs in the whey from that which takes place in the curd, with the result that active acid is produced in whey and the racemic variety in the curd. To settle this point, whey and curd were investigated for the forms of lactic acid occurring in them. The results are given in Table XVII:

TABLE XVII.—Analysis of whey and curd, showing the quantity of lactic acid as zinc lactate

Fraction No.	Whey when drawn.		Curd at hooping time.	
	Crystals of zinc lactate.	Water of crystallization.	Crystals of zinc lactate.	Water of crystallization.
	Grams.	Per cent.	Grams.	Per cent.
1.....	0.4570	13.65	0.1974	12.36
2.....	1.6520	12.50		

Table XVII shows that the whey and curd contained active lactic acid and had a similar course of fermentation during the very early stages. A different fermentation evidently took place in the cheese after pressing, but not in the curd stage. The next question that arose was, At what stage in the curing process did the production of racemic lactic acid take place? The data in Table XVIII show that racemic acid begins to appear very soon after going to press.

TABLE XVIII.—Analysis of whey, curd, and cheese, showing the quantity of lactic acid as zinc lactate

Fraction No.	Whey when drawn.		Curd at hooping time.		Whey kept at temperature of curd and stood overnight in pressing room.		Cheese 24 hours old.		Cheese 48 hours old.	
	Crys- tals of zinc lactate.	Water of crys- talliza- tion.	Crys- tals of zinc lactate.	Water of crys- talliza- tion.	Zinc lactate.	Water of crys- talliza- tion.	Crys- tals of zinc lactate.	Water of crys- talliza- tion.	Zinc lactate.	Water of crys- talliza- tion.
	Grams.	Per ct.	Grams.	Per ct.	Grams.	Per ct.	Grams.	Per ct.	Grams.	Per ct.
1.....	0.2360	13.64	0.3044	13.00	0.6435	13.05	0.0533	17.07	0.1123	17.45
2.....	.0000		.0000		.8010	13.54	.0845	17.37	.1420	17.11
3.....					.2745	13.47	.1260	17.77	.5805	17.07
4.....					.3861	12.96	.2606	14.19	.0965	14.69
5.....					.2788	13.12	.4948	13.29	.4555	13.63
6.....					.0975	13.53				

Whey when drawn, and also after standing overnight, contained active lactic acid. Curds at hooping time contained active lactic acid. One-day-old and two-day-old cheese contains a mixture of racemic and active acid.

The causes for the early production of racemic acid and the disappearance of active acid may be ascribed to a direct production by either enzymes or bacterial action of active acid which is of opposite polarity from that already present.

In order to study the relation of enzymic action in curd to this problem, the following experiment was performed: Curd at hooping time was divided into five parts. The first portion was immediately analyzed for lactic acid; the second portion was analyzed after standing 46 hours in the pressing room; the third portion was kept for 17 days at 35° C.; the fourth part was treated with chloroform and kept for 17 days at room temperature; the fifth portion was treated with chloroform and stood for 3 months at room temperature. The data secured on the nature of the lactic acid produced are shown in Table XIX.

TABLE XIX.—Analysis of curd, showing lactic acid as zinc lactate

Fraction No.	Curd at hooping time.		Curd kept 46 hours in pressing room.			Curd kept 46 hours at 35° C.		Curd kept 17 days with chloroform.		Curd kept 3 months with chloroform.	
	Zinc lactate.	Water of crystallization.	Zinc lactate.	Water of crystallization.	Zinc oxide in lactate.	Zinc lactate.	Water of crystallization.	Zinc lactate.	Water of crystallization.	Zinc lactate.	Water of crystallization.
	Grams.	P. ct.	Grams.	P. ct.	P. ct.	Grams.	P. ct.	Grams.	P. ct.	Grams.	P. ct.
1.....	0.1902	12.92	0.5601	15.97	33.8	0.5515	18.00	0.5517	12.96	0.1490	17.92
2.....	.0000		.5854	14.14	33.9	.6040	18.11	.0000		.2183	14.13
3.....			.2314	13.48		.3901	17.86			.2119	12.79
4.....			.0000			.0000				1.0695	12.78
5.....										.7685	
6.....										.7074	13.21
7.....										.1546	13.06

\* In order to verify the results secured on the increase of racemic acid and the decrease of active acid in fresh cheese curd, as shown above, another sample of fresh curd was divided into three portions. One portion was examined immediately for lactic acid, another after 24 hours, and the third portion after keeping at 60° for 48 hours. See Table XX.

TABLE XX.—Analysis of curd and fresh cheese, showing lactic acid as zinc lactate

Fraction No.	Fresh curd.			One-day-old cheese.			Two-day-old cheese.		
	Zinc lactate.	Water of crystallization.	Zinc oxide.	Zinc lactate.	Water of crystallization.	Zinc oxide.	Zinc lactate.	Water of crystallization.	Zinc oxide.
	Grams.	Per cent.	Per cent.	Grams.	Per cent.	Per cent.	Grams.	Per cent.	Per cent.
1.....	0.2704	12.52	33.6	0.1759	17.19	32.7	0.1070	17.25	33.0
2.....	.0617	11.80	33.4	.0975	12.20	33.3	.0157	17.07	35.2
3.....	.0000			.0778	12.90	33.9	.1240	12.90	33.8
4.....				.1574	12.30	33.8	.2279	13.50	35.9
5.....				.2133	12.00	33.6	.0555	12.00	34.0
Total.	.3321			.7180			.7096		

The theoretical percentage for water of crystallization in racemic zinc lactate is 18.18; in active zinc lactate it is 12.85 per cent. The theoretical percentage of ZnO in anhydrous zinc lactate is 33.3.

From Table XX it is clear that in fresh curd, which contains active lactic acid, the production of racemic acid begins after about 24 hours at room temperature and that this production is accelerated by a temperature of 35° C. In the curd kept with chloroform for 17 days the production of racemic acid appeared to have been checked, while curds kept with chloroform for 3 months gave a small amount of the racemic variety. A parallel case was found by Saiki (1909) in the autolysis of a normal dog's liver, even in a strictly sterile solution. In his experiments racemic lactic acid gradually formed. This enzymic action may be considered a partial cause of the appearance of the racemic acid. The question whether enzymic action decomposes the lactose of the curd

into racemic acid or causes a production of an active acid of opposite polarity to the acid already present has not been settled. It has been shown (Hastings, Evans, and Hart) that the *Bacterium lactis acidii* does produce enzymes, and it may be that these enzymes are one of the factors in the production of racemic acid, although it is more probable that, because of the very slowness of the enzymic action, the real factor is an increasing number of active bacteria of different types from the *B. lactis acidii*.

The question whether *B. lactis acidii* or its enzyme is the cause of the disappearance of active lactic acid and the appearance of racemic acid must be considered. It is known that lactic acid isolated from a lactose solution inoculated with *B. lactis acidii* is active in variety and not racemic. Even after prolonged standing, the lactic acid is found to be active. For this reason it is not believed that *B. lactis acidii* is the direct cause of this change. To determine whether the enzyme of *B. lactis acidii* is the cause of this transformation, a solution containing active lactic acid, formed by inoculation with this organism and after several days treated with toluol, was allowed to stand for 2 months at 35° C. At the end of this time all of the lactic acid isolated was found to be active in variety, as shown by Table XXI.

TABLE XXI.—Analysis of a solution of toluolated active lactic acid, showing active lactic acid

Fraction No.	Zinc lactate.	Water of crystallization.
		Grams. Per cent.
1.....	0.1148	12.98
2.....	.4812	12.96
3.....	1.0186	12.99
4.....	1.3713	13.06

Further, it was thought possible that the kind of lactic acid produced by *B. lactis acidii* might be influenced by temperature conditions. In order to test this, a lactose solution containing 3.6 per cent of lactose, 1 per cent of peptone, and 10 grams of calcium carbonate to 300 c. c. of the solution was inoculated with this organism and put in the ice box. After 38 days the lactic acid isolated was found to be active in form (0.1303 gram of zinc lactate gave 13.04 per cent of water of crystallization); hence it is clear that low temperature does not change the direction of the reaction.

The foregoing experiments lead to the conclusion that the *B. lactis acidii* examined or its enzyme, either in the presence or absence of anti-septics, is not the direct cause of the disappearance of active lactic acid and the appearance of racemic acid. Probably the same conclusion is applicable to cheese curd.

## ACTION OF OTHER GROUPS OF BACTERIA

Consideration must now be given to organisms other than *Bacterium lactis acidii* as an explanation of the change in optical activity of lactic acid in cheese.

A yellow *Micrococcus* was isolated from cheese and inoculated into a lactose solution containing 5 per cent of lactose, 1 per cent of peptone, and 10 grams of  $\text{CaCO}_3$  to 300 c. c. of solution. After 48 hours of incubation, toluol was added. According to analyses made 72, 82, and 105 days after adding the toluol, the quantity of lactose remained constant.

Therefore, the yellow coccus, in this case at least, had no enzymic action on lactose in the presence of toluol. No lactic acid could be isolated from media similar to the above, inoculated with the same coccus, and incubated without antiseptics. This is further corroborative of the fact that the coccus group, as a group, is not a lactic-acid producer and consequently could have no large part in the lactic-acid changes observed in the curds.

In order to ascertain whether the presence of the *Micrococcus* has some influence on *Bacterium lactis acidii* in the latter's action on milk sugar, a mixture of the two bacteria was inoculated into a lactose solution containing peptone and calcium carbonate. The results show that active acid was produced, but not racemic acid, as 3.45 grams of zinc-lactate crystals were obtained, containing 12.96 per cent of water of crystallization. It should, however, be remembered that in this group were found two strains (Tables VIII and IX) which could produce lactic acid on milk media. From the above experiment, where a *Micrococcus* was inoculated alone into a lactose solution, no lactic acid was obtained, but when this organism, presumably the same one, was inoculated into milk a quantity of lactic acid was produced. The ether extract from 300 c. c. of milk which had been inoculated with the organism gave over 100 c. c. of N/10 acidity. Upon neutralizing with  $\text{Ba(OH)}_2$  a voluminous precipitate occurred. The filtrate from this precipitate required about 40 c. c. of N/10  $\text{ZnSO}_4$  solution to take up the barium present. The solution of zinc salts was evaporated to a small quantity and allowed to stand in an ice box for crystallization. There was obtained 0.1031 gram of crystals, which contained 17.53 per cent of water of crystallization. As this is very close to the theoretical percentage of water of crystallization in racemic zinc lactate (18.18 per cent), very nearly all of the lactic acid thus formed was racemic. It would be easy to infer that the organisms from the coccus group discussed above were able to produce very different end-products with some variation in the nature of the media. It is, however, more than probable that the organisms dealt with were distinct in type and physiological action and that the second coccus discussed was one of the strains capable of producing lactic acid.

The next organisms investigated with respect to forms of lactic acid produced were those which belong to the *Bacterium casei* group and which produce a much higher acidity than *B. lactis acidii*, although they grow more slowly than the latter. Several solutions of sterile milk and calcium carbonate inoculated with a culture of *B. casei* gave, on incubation, levo-lactic acid, although Heinemann (1909), experimenting in the same direction with members of this group, obtained racemic acid. Another culture which was isolated from milk gave dextro acid instead of the levo form. These were evidently two different varieties of *B. casei*. They will be designated "*Bacterium casei* 1" and "*Bacterium casei* 2."

*Bacterium casei* 2 was inoculated into 250 c. c. of sterile milk. Calcium carbonate was added, and the medium was allowed to stand for 7½ months. The liquid of the medium had almost evaporated at the end of that time, and crystals had deposited on the bottom of the flask. These crystals were purified by repeated crystallization and then dried in the desiccator. In the resulting product was found 18.07 per cent of calcium, the theoretical percentage of calcium being 18.34 for anhydrous calcium lactate. Rough isolation of lactic acid gave about 0.900 gram of zinc-lactate crystals. Fractional crystallization proved that it was active, the salt being levo-rotatory—that is, the free acid was dextro-rotatory.

Pure cultures of *Bacterium lactis acidii* and *B. casei* and mixtures of these two cultures with and without calcium carbonate were examined, to determine the types of lactic acid present.

Lactic acid was isolated. Its zinc salt was fractionally crystallized, and the water of crystallization was estimated in each fraction of crystals. See Table XXII.

TABLE XXII.—Analysis of 300 c. c. of sterilized milk, showing production of lactic acid by *Bacterium lactis acidii* and *Bacterium casei* 1

Fraction No.	I. <i>Bacterium lactis acidii</i> .		II. <i>Bacterium lactis acidii</i> and <i>B. casei</i> 1.		III. <i>Bacterium lactis acidii</i> and <i>B. casei</i> 1 with calcium carbonate.		IV. <i>Bacterium casei</i> 1.	
	Zinc lactate.	Water of crystallization.	Zinc lactate.	Water of crystallization.	Zinc lactate.	Water of crystallization.	Zinc lactate.	Water of crystallization.
	Grams.	Per cent.	Grams.	Per cent.	Grams.	Per cent.	Grams.	Per cent.
1.....	1.66	.....	1.47	18.06	4.40	18.36	2.54	12.87
2.....	.73	13.36	.02	17.25	3.30	12.75	.16	12.88
3.....	.....	.....	.06	17.95	3.00	13.24	.16	12.89
4.....	.....	.....	.....	.....	3.00	12.90	.....	.....
5.....	.....	.....	.....	.....	.34	12.26	.....	.....
6.....	.....	.....	.....	.....	.65	11.98	.....	.....
	Levo-salt.				Dextro-salt.		Dextro-salt.	

*Bacterium lactis acidii* produced levo zinc lactate, as has already been shown, and *B. casei* 1 gave dextro zinc lactate. The mixture of the two gave racemic acid, as shown in section II. It may be that in curing cheese after pressing, factors similar to those used in these experiments are operative in the production of the racemic variety of lactic acid.

The data in section III, which were obtained from the mixed culture of *Bacterium lactis acidii* and *B. casei* containing calcium carbonate, show racemic zinc salt and also active acid, the latter being produced, no doubt, by *B. casei* 1, the activity of which continues after that of *Bacterium lactis acidii*.

Another experiment, with *Bacterium casei* cultures 1, 2, and 3, the last-named being a pure culture supposedly of a different type from 1 and 2, was carried out as in the previous experiments. The results are given in Table XXIII.

TABLE XXIII.—Analysis of medium consisting of 200 c. c. of sterile milk and 6 grams of calcium carbonate, showing the production of lactic acid from *Bacterium casei* 1, 2, and 3

Fraction No.	Culture 1.		Cultures 1 and 2.		Culture 3.	
	Zinc lactate.		Zinc lactate.		Zinc lactate.	
	Grams.	Water of crystallization.	Grams.	Water of crystallization.	Grams.	Water of crystallization.
1.....	3.40	12.91	13.42	18.00	2.12	12.94
2.....	.32	13.03	.71	16.14	4.19	15.12
3.....	.28	13.00	.64	14.09		
4.....	.00		.00			
	Levo-salt.		Dextro-salt.		Dextro-salt.	

The data show that the mixture of *Bacterium casei* 1 and 2, which in pure cultures produce the two different active lactic acids, gives racemic acid with a slight excess of levo-acid produced by culture 1. This phenomenon might also take place in cheese ripening, producing racemic acid. *Bacterium casei* 3 produces dextro zinc lactate just as culture 1 does, and it also produces the same kinds of volatile fatty acids. Therefore, culture 3 may have been identical with culture 1.

From the foregoing experiments it may at least be concluded tentatively that the formation of racemic lactic acid in Cheddar cheese soon after going to press is due to the later development of organisms of the *Bacterium casei* group principally, together with the possibility that certain forms of the coccus groups can likewise produce racemic lactic acid.

#### SUMMARY

(1) Representatives of the coccus groups of organisms isolated from Cheddar cheese when grown in milk produced large quantities of the volatile acids, particularly acetic acid. These acids were produced from



citric acid or lactose or protein, as the medium was practically free from fat. These organisms did not produce formic acid. As they are present at times in very large numbers in cheese, they, no doubt, produce much of the volatile fatty acids which arise during the ripening process.

(2) One of the strains of *Streptococcus b* was found to produce comparatively large quantities of alcohols and esters—bodies which contribute in a large degree to the flavor of cheese.

(3) A dilute solution of acetic acid and alcohol formed esters by mere contact, without bacterial action. In cheese, however, the dilution is probably too great for this manner of ester formation.

(4) Lactic acid was generally not formed by the coccus groups.

(5) The representatives of the *Bacterium casei* group examined gave results differing from those obtained from the coccus forms. They produced no formic acid, but did form some propionic and much acetic acid.

(6) These organisms produced a large quantity of lactic acid, both active and racemic, and decomposed the citric acid of the media.

(7) Cheese made from chloroformed fresh milk did not yield any volatile fatty acids, showing that inherent milk enzymes are not capable of producing these bodies in any appreciable quantity.

(8) Representatives of both the coccus and *Bacterium casei* groups were able to produce ammonia from milk.

(9) Whey and fresh curds contained active lactic acid. Cheese 1 day old contained a mixture of active and racemic lactic acids.

(10) The cause of the disappearance of active lactic acid and the appearance of racemic acid may be due to enzymic action, combined with the action of those bacteria which can produce both kinds of acid.

(11) Some representatives of the *Bacterium casei* group produced levo lactic acid and others dextro lactic acid from milk. A mixture of these two varieties produced racemic lactic acid. A mixture of *B. lactis acidi* and a levo-producing member of the *B. casei* group gave racemic and active lactic acid. The active acid was probably the result of the longer continued activity of *B. casei*.

(12) Racemic lactic acid found in curing cheese may therefore be produced in a small degree by enzym action, but more probably by the combined action of *Bacterium lactis acidi* and the organisms of the *B. casei* group.

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## CITRUS-ROOT NEMATODE

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### INTRODUCTION

Our ignorance concerning nematodes in general and soil-inhabiting nematodes in particular is well illustrated by the history of the Citrus-root parasite *Tylenchulus semipenetrans*, which, within a few months of its discovery in California, has been located in such widely separated places as Florida, Spain, Malta, Palestine, and Australia. This series of events is, the writer believes, simply illustrative of the surprises in store whenever the soil-inhabiting nematodes receive at the hands of agricultural scientists the attention they merit.

Nematodes are distributed far and wide in inconceivable numbers<sup>1</sup> and without doubt constitute a group in the animal kingdom comparable with insects both in number of species and economic importance.

### THE CITRUS-ROOT NEMATODE *TYLENCHUS SEMIPENETRANS*

The anatomical features of *Tylenchulus semipenetrans*<sup>2</sup> are so well set forth in the accompanying illustrations that it is unnecessary to describe them further. It is well to add that the drawings are so carefully made<sup>3</sup> that many features set forth in them can not be seen in the natural object, except with the aid of the best immersion lenses skillfully used under favorable conditions.

*Tylenchulus semipenetrans* (fig. 1) was first discovered in California on Citrus roots by J. R. Hodges, Horticultural Inspector for Covina County, Cal., and was first carefully studied by E. E. Thomas, of the Agricultural Station of the University of California, at Berkeley.<sup>4</sup>

<sup>1</sup> Cobb, N. A. Antarctic Marine Free-Living Nematodes of the Shackleton Expedition. Contributions to a Science of Nematology.—I. 33 p., illus. Baltimore, 1914.

North American freshwater Nematodes. Contributions to a Science of Nematology.—II. 65 p., 80 fig., 7 pl. 1914. Also published in Trans. Amer. Micros. Soc., 1914.

<sup>2</sup> Cobb, N. A. Notes on Mononchus and Tylenchulus. In Jour. Wash. Acad. Sci., v. 3, no. 10, p. 288, 1913. This article contains the writer's diagnosis of the new genus *Tylenchulus* and its type species, *T. semipenetrans*, Cobb.

<sup>3</sup> The drawings were prepared under the author's direction by Mr. W. E. Chambers, of the Bureau of Plant Industry.

<sup>4</sup> Thomas, E. E. A preliminary report of a nematode observed on Citrus roots and its possible relation with the mottled appearance of Citrus trees. Cal. Agr. Exp. Sta. Circ. 85, 14 p., 8 fig., 1913 (J. R. Hodges mentioned).

Since that time the writer has given some attention to the anatomy, life history, and distribution of this species, and in cooperation with the Office of Foreign Seed and Plant Introduction, Bureau of Plant Industry, and its correspondents abroad, notably the American consuls stationed in Citrus-growing regions, has shown that it is probably world-wide in

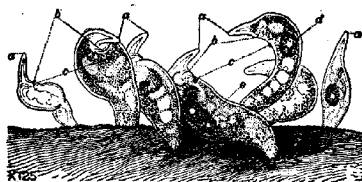


FIG. 1.—*Tylenchulus semipenetrans*: Mature and half-grown females, with their head ends permanently embedded in the feeding root of a citrus tree. This is a plant parasite similar in many ways to the notorious gallworm *Heterodera radicicola*. a, Tail end; b, vulva; c, excretory pore; d, e, egg in uterus.

its distribution. Samples of the feeding roots of Citrus trees, such as *Tylenchulus* infests (figs. 2, 3), were sent to these various foreign correspondents, with the request that they forward to Washington from their localities similar roots taken from trees that appeared to be suffering from malnutrition—

trees that were off color. These roots on being received at the Department were examined, with the result that this Citrus parasite was found to infest Citrus roots at Valencia, Spain, and at Malta. Previously, the nematode had been found in small numbers in one locality in Florida. Through the courtesy of Mr. Charles O. Chambers, of Gosford, New South Wales, Australia, roots of Citrus were obtained from his locality. These proved also to be *Tylenchulus*-infested. Similarly, roots of Citrus sent from Haifa, Palestine, through the courtesy of Mr. Aaron Aaronsohn, were found infested. Particular mention should be made of the numerous specimens of roots of Citrus and soil received from Mr. R. S. Vaile, Horticultural Commissioner for Ventura County, Cal., as well as from Messrs. J. W. McLane and R. L. Piemeisel, of the Bureau of Plant Industry. The evidence shows this parasite to occur in widely separated parts of the world, and it probably occurs in every region where Citrus trees have been successfully grown for any considerable length of time.

It appears also that *Tylenchulus semipenetrans* is peculiar to the feeding roots of Citrus trees. It has never been found attacking any other species of roots, although careful search has been made for it on a variety

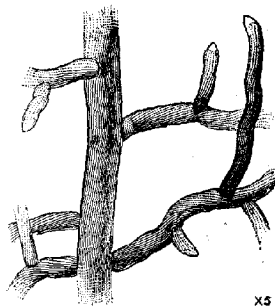


FIG. 2.—Healthy Citrus root magnified 5 diameters. Note the size and form of the healthy root endings. Compare with figure 3.

of plants from Florida and also a variety of plants collected in the vicinity of Citrus groves in California by Drs. L. J. Briggs and H. L. Shantz, of the Bureau of Plant Industry, both of whom have taken an active and exceedingly helpful interest in the nature of the *Tylenchulus* disease, on account of its possible bearing on malnutrition of Citrus trees, a subject to which they are giving special attention. Hundreds of samples of roots of a great variety of plants other than Citrus, collected from various parts of the world, have been examined by the writer without disclosing any specimens of *Tylenchulus*.

We may take it as fairly well established that *Tylenchulus semipenetrans* is a parasite peculiar to Citrus roots, occurring in all parts of the world where Citrus trees have long been grown.

In searching for *Tylenchulus semipenetrans* care must be taken not to confuse it with other species of nematodes. Fortunately its characters are so very well marked that there is very little difficulty in establishing its identity if adult females can be found (see fig. 1). On the other hand, unfortunately, its larval forms closely resemble those of certain other species of nematodes—so closely, indeed, that they can not be identified with certainty, except with the aid of an oil-immersion lens skillfully used by a person conversant with the characteristics of the various genera and species of nematodes. Much the same may be said of the males, as these also are rather exceptionally hard to identify with certainty. The fitness of these remarks will appear when it is stated that already the writer's examinations, directed primarily at *Tylenchulus semipenetrans*, have revealed the presence about the roots of Citrus trees in various parts of the world of toward 100 different species of nematodes, many of which are spear-bearing and have larval forms which might easily be confused with those of *Tylenchulus* by one unskilled in the art of identifying nematodes species.

Whenever larval nematodes from near roots of Citrus are being examined with the object of ascertaining whether or not they belong to the species *Tylenchulus semipenetrans*, the observer should keep in mind that

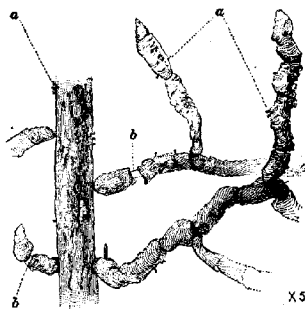


FIG. 3.—Citrus root attacked by the parasitic nematode *Tylenchulus semipenetans*, magnified 5 diameters. The parasites are shown at *a*. They are shown black, but in reality are yellowish or brownish. Compare with figure 2, and note that owing to the presence of the parasite the feeding roots may become somewhat enlarged and irregular and that the outside portion of the root is somewhat separated from the axial portion, as shown at *b*. When the roots are agitated in water, the outside portion sometimes becomes loosened in segments which will slide on the axial portion *b*, somewhat as heads slide on a string.

the development of the male of *Tylenchulus* is of a somewhat peculiar character, in that as it increases in age, it may decrease somewhat in size, and that the oral spear, so characteristic a mark of the group of genera

to which *Tylenchulus* belongs, deteriorates so markedly in this instance that adult males are sometimes found that appear to have no trace whatever of this organ. In all cases it deteriorates so much as to fade into an inconspicuous feature of the anatomy (fig. 4). It is somewhat doubtful whether the males enter the unimpaired tissues of Citrus roots. In fact, most of the evidence appears to point the other way and seems to indicate that they seldom, if ever, enter sound roots. The males mature rapidly, and there is some doubt whether they feed at all; for, as already stated, instead of increasing in size as they grow older, they decrease and become more slender. They are probably ill-fitted to bore their way into the tissues of Citrus roots, lacking, as they apparently do, an efficient puncturing organ. All specimens of *Tylenchulus* seen by the writer to be embedded in comparatively sound roots have proved to be females, though it is entirely possible that an examination of a larger number of cases might prove that males also embed themselves.

The eggs of *Tylenchulus semipenetrans* are of comparatively large size, thin-shelled, and usually are not deposited until after segmentation begins. Their size is such that the uterus of the adult female commonly contains only one or two at a time (figs. 1 and 5); and, as a rule, these are found in the early stages of segmentation, sometimes containing only one blastomere, sometimes two, sometimes three, occasionally as many as a dozen. The exact length of time the eggs remain in the uterus is at present unknown, but under favorable conditions does not exceed a few days. They are deposited one at a time in batches of a dozen to a score or more and are sometimes

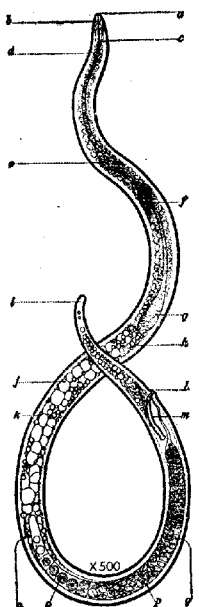


FIG. 4.—*Tylenchulus semipenetrans*: Lateral view of full-grown male. The spear is usually very inconspicuous—always deteriorated. Note also the deteriorated median bulb, sometimes apparently absent. In the male, in contrast with the female, the anus develops. a, Lip region; b, spear; c, 3-bulbed base of spear; d, esophageal lumen; e, median esophageal bulb; f, nerve ring; g, cardiac esophageal bulb; h, beginning of the intestine; i, large intestinal granule; j, small intestinal granule; k, anus; l, spicule; m, excretory pore; n, spermatoocyte; o, vas deferens; p, spermatozoon.

found encased in a somewhat indefinite mass of "gummy" matter. Judging from the experiments, these eggs hatch very promptly, probably within a day or two after being deposited, and produce colorless

larvæ of the form shown in figures 6 and 7. larvæ are slow and weak, and yet the young stages, especially those of the female, are more active than those of the adult. At no stage of its existence are the movements of *Tylenchulus semipenetrans* anything but relatively slow and weak, and it is altogether improbable that through its own muscular exertions it ever migrates any great distance. Once the head of the female becomes well embedded in a Citrus root, in the manner shown in figure 8, c, it is practically impossible for her to retreat. She therefore becomes fixed for life and dies at the point where this entrance was made. "Shells," or empty skins, of dead females are not infrequently found on infested roots. Figure 8, c, shows a female that has partly penetrated a Citrus root. It will be seen that at the surface of the root the body is constricted, both the portion inside and that outside having a much greater diameter (see also fig. 9). It is the swollen character of the anterior portion of the embedded female that prevents retreat. As she grows older and increases in size, her head penetrates farther and farther, but never so far as to be out of harmony with the specific name "*semipenetrans*." By the use of her strongly developed oral spear, the tissues of the roots are punctured, and the food, consisting of the sap and protoplasmic matter of the root, is ingested.

The females of *Tylenchulus semipenetrans* sometimes appear to be somewhat gregarious; at least it is not uncommon to find the adult females arranged in rather definite groups on the roots. The cause of this phenomenon is not yet understood but may, perhaps, arise in this way: Larvæ from the same batch of eggs in searching for food naturally seek the nearest suitable root. The mother worms, having injured or killed the root in their immediate vicinity and the root having accord-

The movements of these

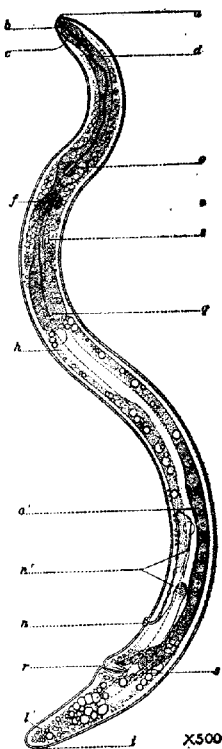


FIG. 5.—*Tylenchulus semipenetrans*: Female ready for fertilization. Note the increased size of the excretory pore, *n*, as compared with previous stages; see figures 7 and 12. The pore is located farther back than in any other known species of nematode. *a*, Lip region; *b*, guide of spear; *c*, shaft of spear; *d*, lumen of oesophagus; *e*, median oesophageal bulb; *f*, nerve ring; *g*, cardiac oesophageal bulb; *h*, beginning of intestine; *o*, ovary; *o'*, duct of renette; *n*, excretory pore; *r*, vulva; *s*, wings of the cuticle; *f'*, degenerated anus; *i*, terminus.

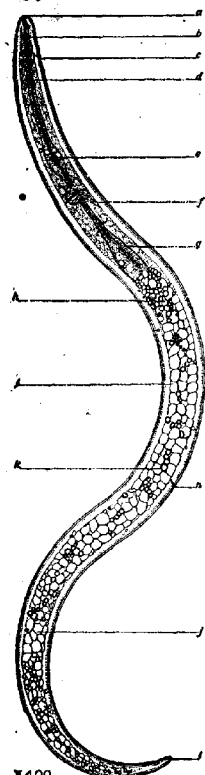


ingly thrown out a new shoot farther back, as often happens in such cases, the new generation of young females make their way to this new shoot. This supposition accords with what is known about the growth of roots in general when injured by disease-producing organisms and is in harmony with the weak muscular powers of the *Tylenchulus*.

Although the movements of *Tylenchulus semipenetrans* are so feeble as to make it seem quite unlikely that it travels any great distance by the aid of its own muscular powers, its small size is in favor of its transportation from place to place in the soil by a great variety of agencies, such as soil water, subterranean insects, worms, and burrowing animals. In this way it may be carried to considerable depths in the soil and doubtless will be found attacking Citrus roots, however deep the latter penetrate. When cultural operations bring *Tylenchulus semipenetrans* to the surface and these cultural operations are followed by irrigation, the eggs and free-living young stages may be carried from tree to tree in the same grove by the irrigation water, or from orchard to orchard, or even occasionally from district to district; in fact, wherever Citrus fruits are grown under irrigation, the irrigation water is undoubtedly one of the principal agents in distributing the pest after it has once become established through the planting out of infested nursery stock.

*Tylenchulus semipenetrans* is comparatively sensitive to temperatures much above those of ordinary soil. When eggs, larvæ, or adults are placed in water above 100° F., they are quickly affected, and at 130° F. are killed. Immersion in water at 140° F. causes almost instant death to all forms of the organism. This fact was demonstrated and utilized in studies of the life history.

To follow the development of the eggs and larvæ, they were placed in capsules constructed in accordance with the explanation accompanying



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FIG. 6.—*Tylenchulus semipenetrans*: Larva, soon after hatching from the egg. Note the absence of the anus. a, Lip region; b, spear; c, bulbous base of spear; d, lumen of the esophagus; e, median cesophageal bulb; f, nerve ring; g, posterior cesophageal bulb; h, beginning of the intestine; i, large intestinal granule; k, small intestinal granule; n, excretory pore; t, terminus.

figures 10 and 11. In the incubation experiments it was thought advisable to give the eggs and developing larvæ conditions as nearly as possible like those in the vicinity of growing Citrus roots. In order that uncertainty might not creep into the experiments, it was only necessary that the material to be placed in the small glass brood capsules be Pasteurized at a temperature of 130° to 140° F. This was done with entire success. Lest any misapprehension arise at this point, it is well to state that not all species of soil-inhabiting nematodes are so sensitive to high temperatures. It is therefore best to use for incubating material soils as free as possible from other species of nematodes.

The form of the brood capsule devised for the incubation experiments is illustrated in figure 11. These glass capsules are easily constructed and manipulated, and, as said before, the hatching and rearing offer few difficulties. The drawing of the capsule is to scale and the explanation is comparatively complete, so that nothing need be added except to say that the material surrounding the egg in the capsule consists of soil particles and Citrus-root detritus, both taken from the immediate vicinity of diseased roots of Citrus. This material, having been Pasteurized, as before described, was used as a nidus in hatching *Tylenchulus* eggs and rearing the larvæ. The capsule was half filled with the nidus, and then the egg to be incubated was introduced by means of a very fine-pointed pipette. As this operation was accomplished with the aid of a magnifying glass of one-half inch equivalent focus, there was proper assurance that no other nematode organism was introduced. The remainder of the nidus was then inserted above the egg and the disk *f* introduced. Afterwards the disk *e* was placed in position. Necessarily, considerable water was intro-

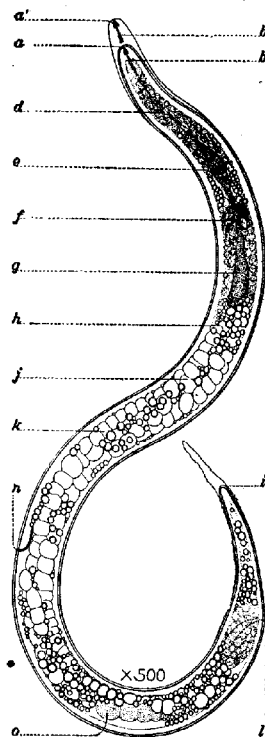


FIG. 7.—*Tylenchulus semipenetrans*: Young larva of male undergoing the first or second molt. Note the deteriorating spear and median bulb, indicating that the males are not so well equipped to penetrate the Citrus roots as the females. The males mature rapidly, perhaps with little or no food. a, Lip region; a', lip region of sloughed skin; b, deteriorating spear; b', spear of the sloughed skin; d, lumen of the esophagus; e, median esophageal bulb; f, nerve ring; g, cardiac esophageal bulb; h, beginning of the intestine; i, large intestinal granule; j, small intestinal granule; k, excretory pore; l, immature internal sexual organ; m, developing male sexual opening; t, terminus.

duced during these operations. Therefore, before the capsule was buried in the soil, it was placed on blotting paper, so that as much water as possible might drain away. This left the capsule in a condition to reabsorb moisture. The soil about the potted Citrus seedling to be used in the experiment was previously allowed to become somewhat dry; in fact, was allowed to go without water until the seedling showed the first signs of distress. The prepared capsule was then placed in the pot adjacent to sound young Citrus feeding roots and the soil replaced in the pot (see fig. 10). The Citrus seedling was then at once watered, so that the capsule was given an immediate opportunity to absorb

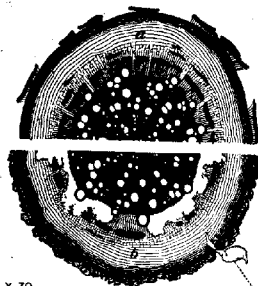


FIG. 8.—Two half-sections of Citrus root magnified 30 diameters: a, Healthy root; b, diseased root. A female specimen of *Tylenchulus semipenetrans* is shown at c with her head end embedded in the root. Note that the outside portion of the diseased root, shown light, is nearly detached from the central axial portion, shown black. Compare with figure 3.

soil moisture that might be described as "of a citrus character."

In the spring of 1913 a number of experiments were started with a view to ascertaining the length of time required or the larvæ of *Tylenchulus semipenetrans* to become adult. For this purpose potted grape-fruit seedlings of a size and character illustrated in figure 6 were used. Material derived from *Tylenchulus*-infested Citrus roots was added to the water supplied to the seedlings. In most cases some of the surface soil was removed, and the infested material applied several days in succession in such a way that there could be no doubt that an abundance of the living larvæ had come in contact with the roots. Previous examinations indicated that few, if any, of the female larvæ added to the water were advanced beyond the earliest stages shown in the illustrations. Check plants were subjected to the same treatment as the infested plants, with the exception that no material containing *T. semipenetrans* was added to the water. There was no difficulty in rearing

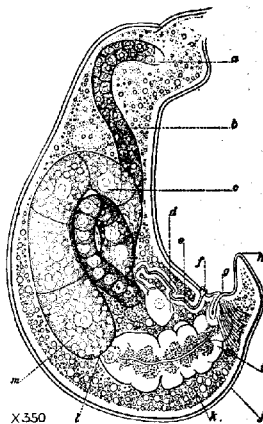


FIG. 9.—*Tylenchulus semipenetrans*: Posterior portion of adult female. The portion shown is located outside of the Citrus root. Compare figures 1, 3, 8, and 13. a, Blind end of the coiled single ovary; b, anterior portion of ovary; c, posterior portion of ovary; d, renette cell; e, duct of the renette cell; f, excretory pore; g, vulva; h, terminus; i, uterus; j, cuticle; k, somatic granule; l, junction of ovary with uterus; m, posterior end of ovary with ovum ready to enter uterus.

*Tylenchulus* by his simple process, and in from six to seven weeks it was possible to secure adult females from roots thus infested.

The following is a typical case: On May 31, 1913, adult females, as well as larvæ and eggs, were removed from the roots of a grapefruit seedling first infested on April 14, 1913. As a rule, the full-grown males were found much earlier than the females; in fact, there can be little doubt that in most cases the infested water supplied at the beginning of the experiment contained a few males that were adult or nearly so. The gist of these experiments is contained in the fact that the infested water would contain but few, if any, females that were advanced beyond the stages shown in figures 12 and



FIG. 10.—Pot containing Citrus seedling. A portion of the wall of the pot has been removed to show location of one of the glass brood capsules shown in figure 11. a, Brood capsule; b, label of same attached to capsule by means of a thread which passes down through the soil. *Tylenchulus* eggs incubated in this way often hatch out in 24 hours. Larvæ reared on grapefruit roots in pots similar to that shown above become adult in six to seven weeks.

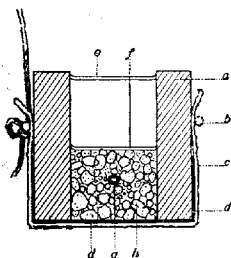


FIG. 11.—Longitudinal section of a glass brood capsule enlarged 10 diameters. The cord at the left leads upward to the surface of the soil. Compare with figure 10. In these capsules the youngest eggs of *Tylenchulus semipenetrans*, even those removed from the uterus, hatch out in 24 to 72 hours when the capsules are placed adjacent to healthy roots of growing Citrus seedlings. a, glass tube shown in longitudinal section; b, thread encircling capsule, shown in cross section; c, finest mesh linen cloth held over bottom of filter capsule by the encircling cord b; d, layer of filter paper, for convenience shown black; e, f, disks of filter paper; g, nematode egg embedded in Pasteurized orange root detritus and soil granules.

13, and that the entire life cycle of the females under the conditions of the experiments was shown to be accomplished in six to eight weeks.

A few words about the probable origin of *Tylenchulus semipenetrans* may not be out of place. As this parasite occurs in so many different, very widely separated Citrus regions and is found only on Citrus roots, it is a fair supposition, as before remarked, that it is peculiar to species of Citrus. The most reasonable explanation of its wide distribution is that it has been sent from point to point in commerce

on the roots of Citrus nursery stock. As this distribution of the parasite has probably been going on for centuries, the obvious surmise is that the original habitat of the parasite is that of the genus Citrus itself.

The original home of the cultivated species of *Citrus* is supposed to be India or southern China, and we may therefore suppose these lands to be

also the original habitat of the *Tylenchulus*.

The life history of *Tylenchulus semipenetrans* strikingly illustrates how little we know about organisms inhabiting the soil. Here is an organism so large as to be visible to the naked eye, an organism that, considered as a microscopic object, is relatively huge, occurring, practically speaking, in most parts of the world where *Citrus* fruits can be profitably grown, attacking and killing the roots of a crop of great economic importance. Yet this organism remained undiscovered for 200 years after mankind possessed instruments for its discovery. With such a striking instance before us, how can we doubt that thoroughgoing biological soil researches will reveal a multitude of similar cases?

It is a common observation among agriculturists that crops fail for some unknown cause. Such cases may appear less mysterious when we have an adequate knowledge of soil-inhabiting organisms.

Not the least interesting feature of these investiga-

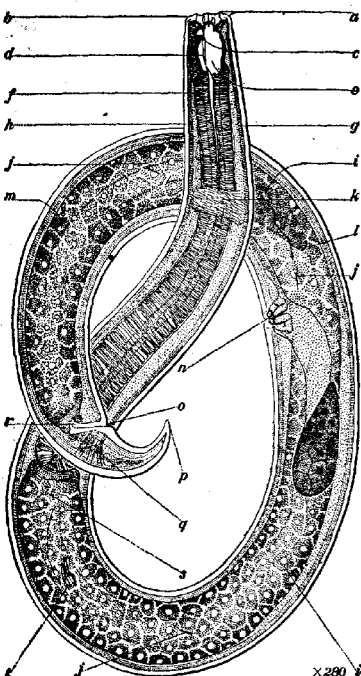


FIG. 12.—*Mononchus papillatus* Bastian: Rather immature female specimen which has been feeding upon *Tylenchulus semipenetrans*. The remains of three or four *Tylenchulus* are to be seen in the intestine. *a*, Two of the innervated papillae existing on one of the six mobile lips; *b*, one of the lips; *c*, dorsal pharyngeal tooth; *d*, one of the three longitudinal chitinous ribs of the pharynx; *e*, pharyngeal cavity; *f*, esophagus; *g*, muscular layer of the body; *h*, cuticle; *i*, one of the cells of the intestine; *j, j, j*, oral spines of three ingested *Tylenchulus*; the spear in the intestine near the vulva is accompanied by an undigested portion of the lumen of the esophagus of the *Tylenchulus*; *k*, nerve; *l*, blind end of the anterior ovary, which, being behind the intestine, shows less clearly than the posterior ovary; *m*, nucleus of one of the intestinal cells; *n*, vulva; *o*, anus; *p*, terminus; *q*, anal muscles; *r*, rectum; *s*, cardia; *t*, spicula of an ingested male *Tylenchulus*. The outlines of the undigested tail end of the male are to be seen faintly.

tions into the life history of *Tylenchulus semipenetrans* has been the discovery of a species of *Mononchus*, another nematode, which regularly feeds upon the *Tylenchulus*, swallowing the males and the larvæ whole. Conclu-

sive evidence on this point is the presence in the intestine of individuals of *Mononchus* of the remains of specimens of *Tylenchulus*. In several such cases no other ingested food appeared to be present, showing that the *Mononchus* had been making a meal on *Tylenchulus*. In searching for the remains of species of *Tylenchulus* in the intestine of a specimen of *Mononchus*, one looks for the more indigestible parts, such as the oral spear and the spicula of the male (see figs. 4 and 6). These being very indigestible and also highly refractive remain visible even when other parts of the *Tylenchulus* have been completely digested.

This *Mononchus* is a relatively large species and, judging from this fact alone, would seem to be capable of devouring *Tylenchulus semipenetrans* in considerable numbers. These discoveries confirm those made earlier by the writer and render it certain that there is a class of beneficial nematodes inhabiting the soil.

The pharynx of the active and predacious *Mononchus* is supplied with a prominent, acute, dorsal, forward-pointing chitinous tooth. This tooth is opposed to six thick, muscular, hookshaped, back-acting lips, and it is by the interaction of these various organs that the *Tylenchulus* is seized, punctured, and killed. Possibly the acute pharyngeal tooth of the *Mononchus* is nothing less than a poison fang, but this at present is only a matter of theory. It is, however, known that many nematodes have glands in the segments of the oesophagus—the so-called oesophageal glands—and it is possible that these glands may secrete a poisonous liquid substance which may be used in the same way as the venom of serpents. Furthermore, the inner surface of the pharynx of the *Mononchus* is sometimes armed with scores of exceedingly minute rasplike teeth, which in some respects resemble, on a small scale, those found in the pharynx of serpents and sharks, teeth which aid

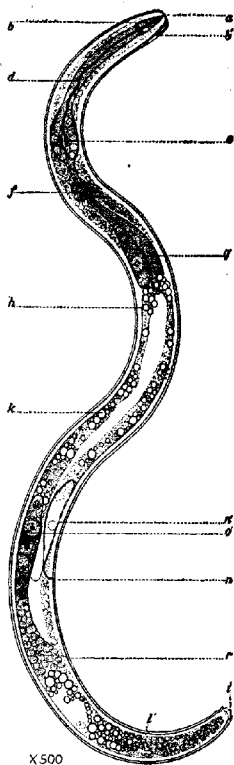


FIG. 13.—*Tylenchulus semipenetrans*: Young female about to undergo its second molt. Compare with figures 1 and 6. Note that the spear in the growing female does not deteriorate. Note also the increasing size of the median or suction bulb of the oesophagus. Quite the contrary occurs in the male. a, Lip region; b, spear; b', spear which has been sloughed off; d, lumen of the oesophagus; e, median oesophageal bulb; f, nerve ring; g, cardiac oesophageal bulb; h, beginning of the intestine; k, larger intestinal granules; n, excretory pore; n', reticulate cell; o', blind end of single immature ovary; r, developing uterus; r', remnant of rudimentary anus; t, terminus.

these latter in swallowing organisms of relatively large size. It is a natural supposition that the minute teeth in the pharynx of the *Mononchus* serve a similar purpose. These minute teeth do not occur in the *Mononchus* here mentioned as feeding on *Tylenchulus*.

The writer has in his possession a specimen of *Mononchus* caught in the act of swallowing another nematode. The *Mononchus* holds its prey in the grip of its "jaws" and partially swallowed. The phenomenon reminds one of the not infrequent discovery of serpents with half-swallowed birds in their maws. The writer has also observed aquatic species of *Mononchus* whose intestines contained only the remains of rotifers, indicating that the species of *Mononchus* do not feed exclusively upon other nematodes.

These definite observations on the *Mononchus* lend new interest to the study of this genus, which is estimated to contain a very considerable number of species, probably a hundred or more when all shall have been enumerated. Upward of 20 species are known to the writer. The examination of almost any good collection of soil-inhabiting or fresh-water nematodes is pretty sure at the present date to reveal one or more hitherto unknown species of *Mononchus*. Both on the ground of the number of species of *Mononchus* and the number of their individuals the matter is one well worthy of further observation.

There are other nematode genera that on structural grounds may now be suspected to be vermivorous, or at any rate carnivorous.

#### CONCLUSIONS

There can be no doubt that *Tylenchulus semipenetrans* is an injurious parasite. There is conclusive evidence that it kills the feeding roots of Citrus trees. The roots die either as a direct result of the attack of this parasite or of the attack of other organisms following in its wake; in other words, the nematode is a primary cause of the death of the feeding roots. Many cases have come under observation in which it was apparent that, had it not been for the nematode, the roots would have remained in a healthy condition. The evidence along these lines is of the same character as that which is relied on in demonstrating injuries due to insects and other macroscopic parasites.

The extent of the damage which may be properly charged up against this parasite is a different matter, and it will be necessary to collect evidence along this line for several years before a final statement can be made. Up to the present the data obtained indicate unquestionably that the investigations should be continued.

The writer's long experience with numerous fungous diseases of the Citrus family has led him to conclude that, though a few of these diseases are very harmful, most of them are of minor importance. There are many fungous diseases of the bark, foliage, and fruit of Citrus trees of such a character that they are easily controlled. In many cases all that is

needed is the careful application of well-known suitable cultural methods. Where the climate, soil, and cultural conditions are suitable to the Citrus family these diseases will not appear, or will appear in such a mild form as to be largely negligible. An examination of the literature of Citrus diseases and of the fungi and other microorganisms which have been found parasitic upon Citrus trees discloses a long list of parasites, so long, indeed, as to suggest that species of Citrus are liable to the attacks of an unusually large number of parasitic microorganisms. The foregoing remarks, of course, apply only to aerial parts of the tree. If, therefore, among these numerous parasites of the above-ground portion of the tree, relatively few are found that are really harmful, the thought arises that something similar may be true of the subterranean parasites. Should this prove true and should *Tylenchulus semipenetrans* prove to be one of a series of rather harmless parasites which attack the roots of these trees, the injurious results of which may be combated by proper cultural methods, then all may be well. This, however, is something that needs to be demonstrated. The history of this parasite is altogether too recent and incomplete to render final judgment possible. Hence the necessity for several years' further careful observation.

It will have been noticed that the evidence thus far accumulated is, in a considerable number of cases, indecisive. It may be compared to a two-edged sword, which may cut in either direction. The parasite is found to be somewhat more abundant in orchards that are out of condition, but so far as the present evidence goes, this may be either because the parasite is there and causing the difficulty, or because the trees are out of condition for some other reason, and therefore are not resistant to the parasite. On the other hand, the parasite is found on the roots of trees which appear to be in good health. Here, again, this may be either because the parasite has not yet been there sufficiently long to cause visible injury or because healthy trees do not have much difficulty in counteracting the bad effects undoubtedly due to the parasite. It would, however, be unwise for Citrus growers to allow such reasoning to lull them into a feeling of security. History abounds with similar cases in which either apathy or optimism has led to deplorable inaction, permitting devastating diseases to continue on their course unchecked.

*Tylenchulus semipenetrans* is distributed from place to place on nursery stock. This is the method by which in all probability it is most frequently distributed from one district to another, and it follows that any method of inspection by which infested nursery stock can be readily distinguished from noninfested will make it possible to establish at least one efficient check on the spread of the disease.

As *Tylenchulus semipenetrans* is a parasite with which from now on Citrus growers will have to reckon, it seems certain that sooner or later appropriate inspection will be undertaken by Federal authorities, by State authorities, and by private enterprise. The most beneficent inspec-



tion is that which results in prevention. To illustrate this point the present conditions in the State of Florida may be cited. An examination of Florida orchards seems to indicate that the parasite is not yet widely distributed in that State; in fact, it has been found in only one locality. If henceforth all nursery stock in Florida is examined for *T. semipenetrans* and no stock is accepted for shipment or planted out unless upon inspection it is found free from this nematode, very much can probably be done to limit the spread of disease.

Hitherto no information has come to light that shows any particular kind of Citrus stock to be more resistant than others. The *Tylenchulus* has been found to occur upon the following stocks: Sour orange, sweet orange, grapefruit, and *Citrus trifoliata*. Investigations are under way with the object of ascertaining whether these stocks vary among themselves in resistance to the *Tylenchulus* and whether it is possible to discover a thoroughly resistant stock.

During the life-history studies it was discovered that hot water is fatal to *Tylenchulus semipenetrans*, and that Citrus roots survive the temperature required to kill the *Tylenchulus*. Some of the smaller roots may be injured, but the larger roots are not seriously harmed, and when the treated trees are planted out, they proceed to grow new feeding roots. These facts lead to the hope that a hot-water treatment may prove to be a more or less efficient means of disinfection or at least prove to be a palliative.

## PRELIMINARY AND MINOR PAPERS

### PELLICULARIA KOLEROGA ON COFFEE IN PORTO RICO

By G. L. FAWCETT,

Plant Pathologist, Porto Rico Agricultural Experiment Station

In a recent publication Dr. J. Kuijper (1912)<sup>1</sup> states that from a comparison of the fungus causing the Zilverdraadziekte of Surinam and the leaf-blight of coffee (*Coffea* spp.) in Porto Rico he is of the opinion that it is not identical with *Pellicularia koleroqa* Cooke of India, judging from descriptions of that fungus, and that it also differs from the fungus causing the candelillo of Venezuela. This conclusion with regard to the identity of the Porto Rican fungus would seem but reasonable if the possession of a gelatinous matrix, such as has been ascribed to *P. koleroqa* in some of the descriptions, were necessary to make it that fungus. However, the fact that errors were made in the original descriptions of other coffee fungi and that in other respects the descriptions agreed fairly well with the appearance of the Porto Rican coffee-blight fungus seemed to justify the writer's referring to it as *P. koleroqa* in one of the reports of the Porto Rico Experiment Station (Fawcett, 1911). Moreover, specimens of *P. koleroqa* which had been collected in Mysore, where Cooke's original specimens were obtained, kindly sent to me by Mr. E. J. Butler, of the Agricultural Institute of Pusa, India, agree in every way with the Porto Rican leaf-blight fungus. It would seem from this that it is a mistake to assume that the Porto Rican fungus was not *P. koleroqa* Cooke.

As to the Venezuelan fungus, studies by the writer cause him to agree with Dr. Kuijper that it is different from the Porto Rican fungus. It is this difference, however, which shows that it is not *Pellicularia koleroqa*. The candelillo of Venezuela is of especial interest, in that Dr. Cooke (1881) identified specimens sent to him at Kew as *P. koleroqa*, as he considered the coffee leaf-blight of the Old and New Worlds to be the same, and consequently all the publications on coffee diseases that have since appeared have similarly treated the subject. This view is in the main correct, but only accidentally so, since, apparently, the Venezuelan candelillo is caused by a related but quite distinct fungus. The appearance of the affected trees, characterized principally by blackened leaves hanging from fungous threads, is the same. But the affected leaves in specimens

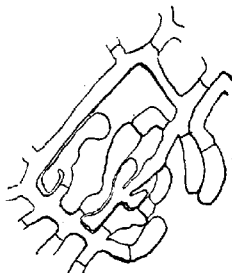


FIG. 1.—Early stage in development of group of hold-fast cells of *Pellicularia koleroqa*.

<sup>1</sup> Bibliographic citations in parentheses refer to "Literature cited," p. —.

gathered in the spring of 1913 were found to be somewhat different, one of the principal differences being the absence of the finely mottled appearance which is taken on at one stage of the disease by leaves affected with *P. koleroga*. This mottling is caused by aggregations of hold-fast cells (fig. 1). In the South American specimens studied such cells are much more evenly distributed and when gathered form in smaller, closer groups.

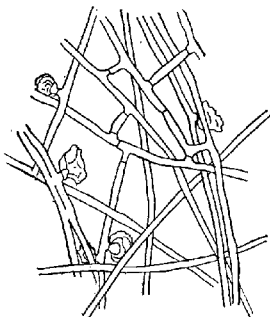


FIG. 2.—Appressoria of Venezuelan candelillo fungus from an old, fully developed specimen.

A further difference is that, instead of consisting of numerous crowded and, in the older ones, overlapping branches originating from various parts of the surrounding mycelium, the hold-fast cells of the Venezuelan candelillo are made up of rather small expansions from isolated short side branches (fig. 2). These fasten the hyphae to the leaf in much the same way as the appressoria of the powdery mildews, to which, however, they bear but slight resemblance, except in arrangement along the hyphae and in function. Still, the earlier classification of the candelillo by Dr. Adolf Ernst (1878,

p. 16) as one of the Erysiphaceae seems less ill-founded if this and not *P. koleroga*, as has been sometimes assumed, were the fungus in question.

The manner of branching and size of the hyphae are the same, but the Venezuelan fungus possesses somewhat thinner or, at least, less conspicuous cell walls, and in places masses of unbranched threads running in all directions are to be found, which are rarely found in the Porto Rican fungus. The differences are such as might be found in closely related species of the same genus. Although the specimens studied were gathered during the dry season and for that reason were not in the best condition, they serve to show that some small but real differences exist between the candelillo of Venezuela and the leaf-blight of India and Porto Rico. In the original description *Pellicularia koleroga* is described as possessing spores, hyaline, echinulate, of about the same diameter as the hyphae, in which they lie without apparent connection. The fact that spores are lacking in the Porto Rican fungus has been taken as additional evidence that it is not *Pellicularia koleroga*. This, however, is a point of little importance. On one leaf of the Indian specimens examined, spores were found which agree with the original description (fig. 3). They had no connection with the larger hyphae, but were seen to be attached to very fine hyphae belonging, appar-

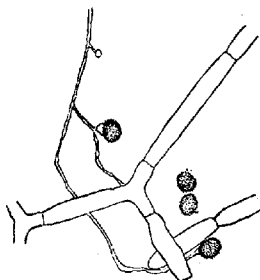


FIG. 3.—Spores of a fungus accompanying *Pellicularia koleroga*.

ently, to some fungus other than *Pellicularia koleroga*—possibly to some of the saprophytes by which it is sometimes accompanied. In some of the Venezuelan specimens, spores occurred which answered fairly closely to the descriptions. Later, an *Aspergillus* was found on some of the leaves which had clearly produced spores on the candelillo-affected leaf. It seems probable that the spores described as belonging to *Pellicularia koleroga* are really those of some other fungus, so that the absence of spores in the species of Surinam and Porto Rico has no bearing on the identity of the fungus, but merely means that the saprophyte producing such spores is not present.

In brief, *Pellicularia koleroga* of India occurs in the Antilles and also on the mainland of South America. Another fungus of similar habit, causing the so-called candelillo, is also found on the continent and is apparently the only fungus of this nature found in those regions of Venezuela in which were collected the first specimens identified, apparently erroneously, as *Pellicularia koleroga* Cooke.

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## FEEDING HABITS OF THE BOLL WEEVIL ON PLANTS OTHER THAN COTTON

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In the course of the investigations on the biology of *Anthonomus grandis* at Victoria, Tex., during the summer of 1913, under the direction of Mr. W. D. Hunter, the writer was able to conduct a number of experiments on the possibility of the boll weevil's breeding in some of the native malvaceous plants. Since the results secured differ with the plants, they are grouped under the various species of plants tested.

The nutritive value of these plants is best shown by a comparison of the longevity of boll weevils fed upon them and the length of life of specimens fed upon cotton and also those kept without food. For this reason the following summary taken from experiments conducted at the same time is given. Forty boll weevils placed on moist sand immediately after emergence and left without food gave a maximum longevity of 6 days, the average for the two sexes being 3.3 days. A number of boll weevils fed only on cotton bolls gave a maximum longevity of 32 days and an average of 17.2 days. Those fed only on cotton leaves had a maximum life of 45 days and an average of 12 days. Of course, the boll weevils fed on cotton squares lived longer than any others. Their maximum life period was 74 days, the average being 40 days.

### FEEDING EXPERIMENTS WITH *SPHAERALCEA LINDHEIMERI*

*Sphaeralcea lindheimeri* Gray is found in small groups on some of the sandy areas near Victoria, though it is comparatively rare. This is evidently the northern part of its range. It is a low-growing, crown-branching plant, and is extremely tomentose throughout. The petals in the buds are very loosely packed and are tightly covered by a heavy, woolly calyx. The buds are very poorly adapted either for the feeding or breeding of boll weevils.

Early in the season six hibernated individuals were collected from cotton in the field and placed with buds, blooms, and fruit of *Sphaeralcea lindheimeri*. These boll weevils fed quite readily, but deposited no eggs. In 22 daily examinations failure to feed was noticed on 5 days. The feeding was never very extensive and was usually confined to the corolla.

The life of these boll weevils after being placed on *Sphaeralcea* was rather short, especially when the amount of feeding is considered. The maximum longevity was 15 days, and the average of both sexes was 8.5 days. It is quite probable that the boll weevils would have been able to live almost as long without any food whatever. The average life of a number of boll weevils collected in the field about the same time and fed on cotton squares was 46.2 days.

Later in the season another experiment was conducted in which boll weevils that had just emerged from cotton squares were placed with the buds, blooms, and fruit of *Sphaeralcea lindheimeri*. There was more

or less feeding in this series almost every day, but it was practically confined to the blooms only.

Twenty boll weevils, ten of each sex, were used in this series, and their longevity was quite regular, ranging from 2 to 8 days, with an average of 4.2 days. This is not quite 1 day above the average for unfed boll weevils; consequently the nourishing power of the plant was not very high. In fact, it is quite doubtful whether the feeding in either series prolonged the life of the boll weevils in the least.

It is hardly probable that the boll weevil will be able to breed in the buds of this plant. The extremely heavy, woolly calyx renders oviposition very difficult, and the contents of a bud are not likely to be sufficient to nourish a boll-weevil larva to pupation.

#### FEEDING EXPERIMENTS WITH CALLIRHOE INVOLUCRATA

*Callirhoe involucrata* Gray is quite common in many parts of Victoria County. In fact, it is the most abundant species of the plants studied during the summer of 1913. Since this plant blooms in the early spring and stops about the first week in June, it was impossible to conduct more than one series of experiments with it as food. Fourteen hibernated boll weevils collected from cotton in the field early in the spring were used. Practically all these boll weevils fed freely on the buds and blooms. Owing to the fact that more boll weevils were introduced later in some of the series, very little accurate information can be given regarding their longevity. The maximum certain longevity was 20 days.

A number of boll weevils were observed in copulation in this series. Two females deposited eggs, one laying two and the other three. These five eggs were placed in four buds. The buds were then placed on moist sand and tested for emergence of adults. Since none emerged, the buds were opened and examined. Three showed no signs of larval work, but one showed that a larva had lived long enough to consume fully one-half of the inner tissue of the bud.

#### FEEDING EXPERIMENTS WITH CALLIRHOE PEDATA

*Callirhoe pedata* Gray is much like the preceding species, but is erect in growth instead of procumbent. It is comparatively rare near Victoria. Rather thorough tests were made of this plant as a food plant for boll weevils. Early in the spring eight hibernated individuals were collected in the field and placed on it. These weevils fed freely on the buds and blooms, but deposited no eggs.

In this series the maximum longevity after collection was 26 days, and the average, 12.1 days. This is considerably above the record of the field-collected boll weevils fed on *Sphaeralcea*, but is still far short of the longevity of the boll weevils fed on cotton squares.

Later in the season 24 boll weevils which emerged from cotton squares were placed with the buds and blooms of *Callirhoe pedata* as food. The longevity record of this series is rather surprising. With the exception of one boll weevil, which lived for 21 days and ate regularly every day, the maximum longevity was 6 days, and the average was about as low as that for unfed boll weevils. In the case of the one exception the sustaining value of the plant is shown clearly, but for some reason the remaining 23 boll weevils were not so well adapted to the food, though they ate heartily during the few days they lived. Including the boll weevil which

lived for 21 days, the average longevity was 4.4 days. In this series no eggs were deposited.

This species of mallow probably ranks about the same as *Callirhoe involucrata* as a host plant for the boll weevil. The buds are smaller, and consequently the chance of breeding is very slight. Both the buds and blooms seem to be of some nutritive value for the boll weevil.

#### FEEDING EXPERIMENTS WITH HIBISCUS SYRIACUS

*Hibiscus syriacus* L. is a large, woody perennial, commonly called "white althea." Quite a number of the plants were found growing in lawns and cemeteries throughout Victoria. Several cultural varieties are found, the chief differences being in the color and form of the bloom. The color varies from pure white, through pink to blue and purple. The most important difference, however, is in the arrangement of the stamens and petals. The latter vary from a single row to a great number very irregularly arranged.

The buds are covered with the tough pilose calyx until they begin to open. Superficially a section cut through a bud shows the interior tissues to be much the same as in cotton squares. There is the same arrangement of the petals and immature anthers.

The foliage is very tough, being so different from the tender, succulent foliage of cotton that the boll weevil could not be expected to feed upon it.

Attention was first attracted to *Hibiscus syriacus* by the fact that on June 16 the writer found a boll weevil feeding on the anthers of a bloom at Victoria. The plant was a large one in the rear of the laboratory and stood about 30 feet from a small patch of cotton which was rather heavily infested with boll weevils. When found, the boll weevil was busily eating the pollen of the bloom and had destroyed almost all of the anthers. Since this was the first record of the species being found feeding on any plant except cotton and *Thurberia* (Arizona wild cotton), it was considered advisable to make thorough tests of the longevity of the boll weevil on *H. syriacus*, and also to determine whether they would breed in the buds. The experiments with this aim may be divided into three series, according to the locality from which the boll weevils were derived.

#### EXPERIMENTS WITH TEXAS BOLL WEEVILS

The first series consisted of Texas boll weevils (*Anthonomus grandis*) either collected in the field or reared from cotton squares in the laboratory at Victoria. Different lots were tested on buds alone, blooms alone, and on buds, blooms, and young fruit together.

In order to test the exact nutritive value of buds alone, one series of 10 boll weevils was started on buds alone. The results from this series were very surprising. Feeding was noted on only 2 days, and the maximum longevity was 5 days, with an average for both sexes of 3.7 days. This length of life is very little above that for unfed weevils, and it is extremely doubtful whether the buds prolonged the life of any of the boll weevils in the least. This is quite in accord with the fact that in all series offering a choice of food there was very little feeding on the buds.

Owing to the fact that in the feeding series where a choice of food was offered the boll weevil fed so very much more on the blooms than any other part of the plant, another experiment was conducted to determine the length of life of boll weevils fed only on blooms from the time of their emergence. Six insects were used, and they fed every day from the



starting of the experiment to the death of the last boll weevil. The longevity was surprisingly great, only one boll weevil dying in less than 24 days, and the average for both sexes being 25.3 days, with a maximum of 40 days. It is evident that the blooms are better food than the buds. The longevity of the bloom-fed boll weevils is much greater than of those fed either on cotton bolls or leaves and compares well with the longevity on squares.

The pollen is the first choice of the boll weevils. One weevil will soon destroy every anther in a large bloom and usually emerges covered with pollen. However, in practically every case there is more or less feeding on the corolla itself. This frequently takes the form of large areas eaten from a beginning on the margin of a petal, but often the petal is merely riddled with small holes.

By far the greatest number of experiments on feeding *Hibiscus syriacus* were series where buds, blooms, and young fruit were offered to the boll weevils every day.

Some boll weevils were reared in the laboratory and placed on *Hibiscus* immediately after emergence, while others were collected in the field and consequently had fed first on cotton.

Three lots of boll weevils collected in the field—12 in all—were used. In two lots they were collected in the field, brought to the laboratory, and were immediately placed in tumblers with a base of moist sand and containing fresh buds, blooms, and young fruit of *Hibiscus syriacus*. In the other lot, hibernated individuals that had been collected some days previously and fed on cotton squares until the time of starting the experiment were used in the same manner. The four pairs collected in the field were in copulation at the time of capture. When possible, the food was changed often enough to give a constant supply of fresh buds, blooms, and young fruit.

The boll weevils all began feeding immediately after being placed with the *Hibiscus*. In a total of 53 examinations feeding was found in all but 2 cases. Both of these were found toward the last of a series when only one boll weevil remained, affording striking evidence of the readiness with which they fed on *Hibiscus* even when accustomed to cotton.

An analysis of feeding by the parts of the plant attacked gives the following: Corolla, 40 times; stamens, 40 times; buds, 14 times; and young fruit, 6 times. This shows the very decided preference for the bloom.

Although the females used in two series were in copulation when collected in the field, only one egg was secured during the experiment. This egg was deposited normally in a bud 31 days after the female had been placed on *Hibiscus*. It hatched, and the larva lived until about half grown. During its life it consumed much of the tissue of the bud.

The maximum longevity was 36 days, the average being 16 days. While this longevity is short when compared with that on cotton, it certainly shows that it is possible for the boll weevil accustomed to feeding on cotton to subsist for a long time on *Hibiscus*.

Three series of boll weevils reared in the laboratory were used in another experiment—one lot in the spring, one in summer, and one in the fall. In all of these experiments the boll weevils were reared on cotton bolls or squares in the laboratory. They were then placed immediately in tumblers containing a layer of moist sand and offered a mixture of buds, blooms, and young fruit every day until the supply of food was exhausted.

The 12 weevils in the spring series fed quite freely and regularly. In a total of 69 examinations feeding was found in all but 3 cases, and 1 of these was when nothing but mature fruit was offered. An analysis of the feeding shows the following: Corolla, 39 times; stamens, 37 times; buds, 27 times; and young fruit, 10 times. While this shows the usual preference for the corolla and stamens, the amount of feeding on buds is unusually large.

Eggs were deposited by each lot of boll weevils, but on only four different days, a total of 15 being found. The maximum number per lot in one day was eight.

The maximum longevity of these boll weevils was 43 days, with an average of 19.2 days. This is above the average for boll weevils fed on either cotton bolls or leaves.

The period from emergence to deposition was 5 days in each series.

Although 15 eggs were deposited in this series, they were distributed in only 4 buds. These were placed in cloth-covered tumblers on moist sand and tested for emergence of adults. When no adults appeared at the proper time, the buds were opened and the contents examined. Of course, it was impossible to determine at that time whether the eggs actually hatched, but if they did, the larvæ died before reaching any considerable size, as there were no signs of larval work in any of the buds.

Only six boll weevils, divided into three lots, were used in the summer series. In a total of 37 examinations feeding was found in all but 8 cases. The feeding by parts of the plant was divided as follows: Anthers, 24 times; corolla, 13 times; and buds, once.

No eggs were found any time, but owing to the extremely dry weather, the buds at this season were not very choice and the supply was not sufficient to have fresh ones always present.

The maximum longevity for the series was 26 days, the average for the two sexes being 14.1 days.

Twelve boll weevils were started in the fall series on September 9 and 10. These were fed on the buds, bloom, and young fruit of the pink variety of Hibiscus, the food being renewed often enough to insure the presence of a fresh supply all the time. This was continued as long as the food was available.

Four females and seven males were used, one male having escaped on the second day of the experiment. Although these boll weevils were not examined more than once a day, each female was observed in copulation at least once at the time of this examination, seven acts of copulation by the four females being observed.

Each of the females deposited at least one egg, the four depositing 19 eggs. The period from emergence to deposition ranged from 12 to 18 days, with an average of 14 days. The period of oviposition varied from 7 to 15 days, excluding the record of one female that deposited only one egg. The average was 11 days.

In a total of 62 examinations for feeding during the period when food was present the feeding was usually quite extensive, and not a single case occurred when there was no feeding. An analysis by parts of the plant attacked gives the following: Stamens, 52 times; corolla, 50 times; buds, 5 times; and pistil, once.

On October 13 the supply of Hibiscus was completely exhausted, and the boll weevils died 4 to 5 days afterwards, the average being 4.4 days.

As a majority of the boll weevils were still alive at the time it was necessary to stop feeding them, no definite longevity can be given, but the following facts will show something of what might be expected. Of the 11 weevils tested, 1 was accidentally killed when 32 days old, 3 died with an average longevity of 30 days, and 7 were still alive at the time of closing the series—35 days after their emergence. From this it is readily seen that the longevity would have been very great had it been possible to continue the series to the normal death of the boll weevils.

All of the 19 eggs deposited were placed in buds, except 1, which was deposited on the inside of the base of a petal during a day when no buds were fed—September 21. This egg was left on the petal, covered with moist cloth, and placed on moist sand. It hatched on September 25—four days later. The larva appeared completely normal. A fresh *Hibiscus* bud was opened to the center with a knife, and the small larva was dropped into a cavity formed there. Then the bud was closed and placed on moist sand. This larva was watched by opening the bud every few days. Unfortunately, it became infested with mites (probably *Pediculoides* sp.) when nearly fully grown—October 3. On October 5 it pupated, but died soon after completing the change. The death was probably due to the attack of the mites, as larvæ in immature stages being reared on cotton squares on the same shelf were killed by them.

The remaining 18 eggs were distributed in 11 buds. Two of these produced adult boll weevils, 4 bloomed and thus prevented breeding, 4 showed no signs of larval work, and 1 gave indications of the larva being alive until it had consumed most of the tissue of the bud.

The two adults that emerged were males. In one case the egg was deposited on September 24, the adult emerging on October 12. In the other the egg was deposited on September 27, the adult emerging on October 14—developmental periods of 18 and 17 days, respectively.

A summary of the spring, summer, and fall series of observations is of interest, in that it shows the conduct of the boll weevils throughout the season when offered their choice of all edible parts of the plant. Table I gives the results of the observations on the preference of the boll weevil for certain parts of *Hibiscus*. It is readily seen that the bloom (stamens and corolla) is very much preferred to all other parts, forming 83 per cent of the total number of times of feeding. That the feeding is quite constant is shown by the fact that in 168 examinations only 11 records of no feeding were made—only 6.5 per cent.

TABLE I.—Summary of feeding experiments of the Texas boll weevil, showing its preference for certain parts of *Hibiscus syriacus*

Series.	Part of plant.				
	Stamens.	Corolla.	Bud.	Fruit.	Pistil.
Spring.....times fed....	37	39*	27	10	0
Summer.....do.....	24	13	1	0	0
Fall.....do.....	52	50	5	0	1
Total.....	113	102	33	10	1

The longevity will be discussed in the general summary. Eggs were deposited in only two series, 34 being found.

## EXPERIMENTS WITH LOUISIANA BOLL WEEVILS

In order to determine whether the feeding habits on *Hibiscus syriacus* of boll weevils reared from cotton at Victoria, Tex., were adaptive habits acquired by long presence there and whether they were peculiar to that locality, a number of cotton squares infested with the same species were imported from Tallulah, La., and the adults reared from them were also tested for feeding and longevity on *Hibiscus*. As blooms only were available, no other food was offered. The four boll weevils used fed very readily on the blooms and were able to subsist on them for very long periods. At the time of closing the experiment, owing to the lack of food, only one out of the four weevils had died, and this one had lived for 21 days. The remainder were still alive and feeding at this time—33 days after starting the first lot and 32 days after the second.

In the 39 examinations recorded, absence of feeding was found only once. The anthers were attacked 32 times and the corolla 18 times.

It is greatly to be regretted that a supply of buds was not available so that tests could have been made of the breeding of these Louisiana boll weevils in them, as the lack of either positive or negative records on this point make the results less definite than they would otherwise have been. But the readiness with which these boll weevils fed on the blooms and their extreme longevity seem to indicate that they are quite as well adapted to *Hibiscus* as those from Texas and quite as likely to breed in it.

The importance of this plant as food for boll weevils is shown by the fact that the three remaining died in an average of 4.6 days after the last day of feeding.

FEEDING EXPERIMENTS WITH *ANTHONOMUS GRANDIS THURBERIAE*

Since *Anthonomus grandis*, var. *thurberiae*, was already adapted to at least one plant other than cotton (*Thurberia thespesiodes*), it was considered probable that it would be able to breed in *Hibiscus* buds. For determining this point a number of these boll weevils which had just emerged from *Thurberia* bolls imported from Arizona were placed on *Hibiscus*. The results of these experiments follow.

A few boll weevils emerging from *Thurberia* bolls were placed with blooms of *Hibiscus* and tested for the longevity and feeding. A number of boll weevils were started in this series, but several lots were killed in the first few days by what seemed to be a bacterial disease. This reduced the number to two, which were carried through the experiment. These boll weevils did not feed nearly so readily nor so much as the Texas or Louisiana boll weevils under observation at the same time. In the total of 21 observations two records of no feeding were made. Feeding on the anthers was noted 18 times and on the corolla, 13 times.

One boll weevil lived 15, the other 30 days, an average of 22.5 days. The number tested was too small for this record to be of much value.

In the second series nine weevils were fed on the buds, blooms, and fruit of *Hibiscus*—five females and four males. In the 40 observations made while food was present there was not a single case of no feeding. The analysis of the feeding shows parts of the plant fed on, as follows: Corolla, 31 times; stamens, 31 times; and buds, 15 times. The usual preference for the blooms is shown.

This series was also interrupted by exhaustion of the food supply. Of course, this prevents a definite statement of their longevity, but the fol-

lowing summary will give an idea of what might have been expected from these weevils. Of the nine boll weevils started, two died with an average longevity of 6 days, while seven were still alive when the food became exhausted—34 days after the emergence. These seven weevils died on an average of 4.8 days after the stoppage of feeding.

Each female was observed in copulation at least once, and a total of 13 records of copulation were made in the course of the daily examination. The first pair observed in copulation performed this act 6 days after emergence.

Thirty-six eggs were deposited, eighteen in each series. The periods from emergence to oviposition were 12 and 14 days in the cases where this record was available.

The eggs deposited were placed in 21 buds, an average of 1.7 eggs per bud. Three of these buds produced adult boll weevils, 8 bloomed, 9 showed no sign of larval work, and 1 was nearly consumed by a larva before its death. The eggs deposited on September 21, 24, and 30 produced a male, a female, and a male on October 8, 10, and 15—developmental periods of 17, 16, and 15 days, respectively.

#### SUMMARY OF HIBISCUS EXPERIMENTS

From the foregoing experiments it is quite evident that it is possible for *Anthonomus grandis* and *A. grandis thurberiae* to breed in the buds of *Hibiscus syriacus*. And not only is this possible, but all indications point toward the conclusion that this breeding would be no rarity. While there was little oviposition and no breeding in the series conducted early in the season, this may have been due to the writer's lack of knowledge of the correct way to keep the food in proper condition. The oviposition in the fall series may seem low, but that of females on squares at this time was no higher. The weather was unusually cold during this period and the oviposition of all boll weevils, regardless of food, was extremely variable by days.

These data prove beyond doubt that the boll weevils fed from the time of emergence only on the buds and bloom of *Hibiscus* can develop sufficiently sexually to produce a number of normal fertile eggs and to deposit them normally. The copulation of these boll weevils was quite normal. Almost every pair was observed in copulation in the course of the daily examination. In a number of experiments in attempting to get weevils to copulate when they had been fed only on cotton leaves since emergence the writer was successful in only a very limited number of cases and was never able to secure a record of any of these depositing eggs, though they lived for long periods. From this it seems probable that some feeding on bud, bloom, or fruit tissue is necessary for sexual maturity, and the buds or bloom of *Hibiscus* will serve this purpose instead of those of cotton.

The eggs deposited were all placed in normally sealed punctures, and dissection of the buds always showed them to be placed partially within the inner folds of the corolla and partially within the outer layer of immature anthers. At least 90 per cent of all the eggs deposited were placed in older buds which had started to open slightly, and the punctures were made through the exposed tips of the involuted corolla. The favorite location of these egg punctures is through the corolla in the base of the clefts then forming between the sepals. This deposition in the corolla is probably due to the extreme pilosity and toughness of the calyx.

However, a few eggs have been deposited in punctures made through the calyx, but in these cases the boll weevils seem to experience great difficulty in sealing the opening. The tissue of the exposed corolla probably most nearly approximates that in which the boll weevils are accustomed to develop. It is of interest to note that of the boll weevils collected from cotton in the field and placed on Hibiscus only one deposited a single egg.

The older buds of Hibiscus are very hardy, and the puncture of the boll weevil very rarely prevents them from opening and shedding the eggs or larvæ. The number of instances of this occurring is readily shown by a glance at the preceding records of tests of the eggs. Although these buds had been picked from the plants and placed in tumblers, a very large percentage of them bloomed and so prevented breeding. This characteristic of Hibiscus, taken in connection with the habits of the boll weevil, is undoubtedly of great importance in preventing it from breeding in the buds. The boll weevil naturally selects the older buds, which are slightly opened at the tips, for oviposition, but these buds are usually able to open and rid themselves of the pest.

The food preference displayed by the boll weevil is quite pronounced. Almost all feeding is on the corolla and the stamens of the bloom. Next to these in importance come the buds and then the young fruit. The latter are so very different in tissue and formation that it is not surprising that boll weevils will not feed on them to any extent. In fact, the only cases of feeding on fruit were when it was young, usually within a day or two of the dropping of the bloom.

While the various series of the Louisiana and Texas boll weevils and the Arizona *Thurberia* weevil were not sufficiently similar to allow an exact comparison, some indication of the extent and nature of the adaptation can be seen. The conduct of the three types of weevils in relation to feeding was practically the same. All showed the same food preference, and, allowing for quite natural variations, the extent of the feeding was much the same. While the experiments with the Louisiana boll weevils were quite limited in extent, they gave all indications of as much adaptation to the food as the native and *Thurberia* weevils.

The longevity of these weevils is of considerable interest. Since the three series under way when the supply was exhausted were by far the most important in this respect, the only figures which can be given are very unsatisfactory. The average longevity of both sexes feeding on buds alone was 3.7 days. That for blooms alone was 25.3 days. As these two series were carried on at the same time and were identical in conditions, the comparison shows the relative food values of the two. Of the boll weevils fed on buds, blooms, and fruit the spring series averaged 19.2 days and the summer series, 14.1 days. However, these records are shown to be of little value when compared with the fall series. The longevity of the three series, interrupted by the lack of food, may be summarized as follows: Of the 24 weevils started on this food, 17 were still alive, 1 was killed when 32 days old, and 6 had died with an average longevity of 20.5 days. This was on October 13, or 33 days after the emergence of the adults used. Both sexes of the boll weevils collected in the field and then fed on Hibiscus died on an average of 16 days after being placed on this food.

The developmental periods of the boll weevils under discussion are shown in Table II.

TABLE II.—Comparison of developmental periods of Texas boll weevils and *Thurberia* weevils in buds of *Hibiscus* and of Texas boll weevils in cotton

Thurberia weevils on Hibiscus.			Texas boll weevils on Hibiscus.			De- velop- mental period for Texas boll weevils in cotton squares at same time.
Eggs deposited.	Adults emerged.	De- velop- mental period.	Eggs deposited.	Adults emerged.	De- velop- mental period.	
		Days.			Days.	Days.
September 21.....	October 8.....	17	September 24.....	October 12.....	18	.....
September 24.....	October 10.....	16	September 27.....	October 14.....	17	.....
September 30.....	October 15.....	15	.....	.....	.....	.....
Average.....	.....	16	.....	.....	17.5	17

From Table II it is seen that there was little difference between the *Thurberia* weevils and Texas boll weevils in the *Hibiscus* buds. The average developmental period of native Texas boll weevils from eggs deposited in cotton squares during this same time is offered for comparison.

## SUMMARY

Table III shows a summary of the longevity of the various series.

TABLE III.—Summary of longevity of boll weevils on various *Malvaceae* tested.

Period of experi- ment.	Food plant.	Series and remarks.	Longevity.			
			Maxi- mum.	Average.		
				Male.	Fe- male.	Weighted.
May 13 to May 28. Do.	<i>Sphaeralcea</i> <i>lindheimeri</i> .	Hibernated boll weevils collected in field.	Days. 15	Days. 7.3	Days. 10.0	Days. 8.5
May 13 to June 2.	do.	Boll weevils reared from cotton squares.	8	4.3	4.1	4.2
May 13 to June 11.	<i>Callirhoe involucrata</i> .	Boll weevils collected in the field.....	20	.....	.....	.....
June 13 to June 11.	<i>Callirhoe pedata</i> .	do.....	26	14.0	10.2	12.1
June 13 to July 11.	do.	Boll weevils reared from cotton squares.	21	5.3	3.6	4.4
June 27 to July 1.	<i>Hibiscus syriacus</i> .....	Reared boll weevils fed on buds only.	5	3.2	4.2	3.7
June 27 to Aug. 5.	do.	Reared boll weevils fed on blooms only.	40	30.0	16.0	25.3
June 16 to July 22.	do.	Boll weevils collected in the field and fed on buds, blooms, and fruit.	35	15.3	16.8	16.0
June 13 to July 23.	do.	Boll weevils reared from cotton squares and fed on buds, blooms, and fruit; spring series.	43	15.1	23.3	19.2
Aug. 28 to Sept. 23.	do.	Boll weevils reared from cotton squares and fed on buds, blooms, and fruit; summer series.	26	9.0	19.3	14.1
Sept. 5 to Oct. 8.	do.	<i>Anticonomus grandis</i> <i>thurberiae</i> reared from <i>Thurberia</i> bolls and fed on blooms only.	30	.....	22.5	22.5

TABLE III.—Summary of longevity of boll weevils on various *Malvaceae* tested—Contd.

Period of experiment.	Food plant.	Series and remarks.	Longevity.			
			Maximum.	Average.		
				Male.	Female.	Weighted.
Sept. 9 to Oct. 18.	<i>Hibiscus syriacus</i> .....	Boll weevils reared from cotton squares and fed on buds, blooms, and fruit; fall series; boll weevils still alive at conclusion of experiment.	Days. 35+	Days.	Days.	Days. 33.5+
Sept. 1 to Oct. 18.	.....do.....	Boll weevils reared from squares imported from Tallulah, La.; fed on blooms only; still alive at conclusion of experiment.	33+			29.7+
Sept. 9 to Oct. 18.	.....do.....	<i>Anthonomus grandis thurberiae</i> reared from <i>Thurberia</i> bolls and fed on buds, blooms, and fruit; still alive at conclusion of experiment.	34+			26.6+
June 18 to July 20.	Cotton.....	Bolls only.....	32	24.0	12.6	17.2
June 9 to July 24.	.....do.....	Leaves only.....	45	15.2	8.8	12.0
June 5 to Oct. 10.	.....do.....	Squares only.....	74	35.3	47.4	40.0
June 22 to July 2.	Unfed.....	On moist sand.....	6	3.4	3.3	3.3

The averages of the three unfinished series are included in Table III for comparison. From these it is seen that the record for native Texas boll weevils on *Hibiscus* is very little short of the final average for cotton-square-fed boll weevils, although the latter were continued to death.

Eggs were deposited in only two plants, *Callirhoe involucrata* and *Hibiscus syriacus*—5 in the former and 71 in the latter. By series those in *Hibiscus* were divided as follows:

Field-collected female, 1 egg; spring series, 15 eggs; fall series, 19 eggs; and *Anthonomus grandis thurberiae* series, 36 eggs.

All experiments were performed under cage conditions, but these were made as nearly normal as possible. No boll weevils have been found breeding in plants other than cotton and *Thurberia* under field conditions, and only one case of feeding under such conditions has been observed. This was in the case of a single boll weevil found feeding on *Hibiscus syriacus*, at Victoria, Tex., on June 16.





## IDENTITY OF PERIDERMIIUM FUSIFORME WITH PERIDERMIIUM CEREBRUM

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In this paper the Cronartium stage of the fungus under discussion is called *Cronartium cerebrum* (Peck), n. comb., in place of the *C. quercus* (Brond) Arthur or *C. quercuum* Miyabe of previous writers. This new combination is made because the authors, after a careful examination of authentic material of the so-called *Cronartium quercus* of Europe, find that it is not the same fungus as the American Cronartium on oaks (*Quercus* spp.).

*Peridermium cerebrum*, the name for the aëcial stage of the American Cronartium on oaks, was published by Peck in 1873,<sup>1</sup> and as this is the oldest specific name for the fungus the combination *Cronartium cerebrum* (Peck) must be used.

Further evidence that *Cronartium quercus* of Europe is not *Cronartium cerebrum* of America is found in the following facts:

(1) Only the uredinial stage of the European fungus has been found. If this rust was the same as the American species, then the telial stage should certainly have been collected, as it follows closely (within 5 to 10 days) the uredinial stage.

(2) No aëcial stage corresponding to *Peridermium cerebrum* has been reported from the European countries where the so-called *Cronartium quercus* is found.

An investigation of *Cronartium cerebrum* and of its aëcial form (*Peridermium cerebrum* Peck) has been conducted by the senior writer for a number of years. As a result of numerous inoculations of several species of pines (*Pinus* spp.) with the telia of this fungus from pedigreed cultures inserted in wounds and made under control conditions in the greenhouse at Washington, D. C., it has been found that in the pines having two needles, or two to three needles, in the cluster, globoid swellings or galls are usually formed on the limbs and twigs, while in pines having three needles in the cluster, fusiform, spindle-shaped, or oblong swellings are commonly found, which are occasionally accompanied by a reversion to the juvenile type of leaves, and by the formation of hexenbesen (witches'-brooms).

In the inoculation experiments mentioned above spheroid swellings were commonly formed on *Pinus contorta*,<sup>2</sup> *P. edulis*, *P. densiflora*, *P. divaricata*, and *P. virginiana*; and fusiform swellings, as a rule, were formed on *P. coulteri*, *P. ponderosa*, *P. radiala*, and *P. sabiniana*. Since fusiform swellings are produced by *Peridermium fusiforme* Arthur and Kern (Pl. XI, fig. 2) and *Cronartium cerebrum* is found throughout the range of this *Peridermium*, these results suggested that it might be identical with *Peridermium cerebrum* (Pl. XI, fig. 1).

In nature the writers have observed that the spheroid galls are usually found on *Pinus divaricata*, *P. clausa*, *P. echinata*, *P. glabra*, *P. resinosa*,

<sup>1</sup> Peck, C. H. Descriptions of new species of fungi. In Bnl. Buffalo Soc. Nat. Sci., v. 1, p. 68. 1873.  
<sup>2</sup> The nomenclature of trees used in this paper is that of Geo. B. Sudworth, U. S. Dept. Agr., Div. Forestry Bul. 14, 1897.

and *P. virginiana*, and that fusiform swellings occur on *Pinus serotina* and *P. taeda*. The former are commonly produced by *Peridermium cerebrum* and the latter by *Peridermium fusiforme*. In the same locality on pines of the 2-needle group the swellings are spheroid and typical of *Peridermium cerebrum*, and on adjacent pines of the 3-needle group they are usually fusiform and typical of *Peridermium fusiforme*.

The junior writer has observed that the fusiform type of swelling (*Peridermium fusiforme*) on *Pinus taeda* is often accompanied by a marked development of hexenbesen (witches'-brooms) at the distal end of the swelling. At Brooksville, Fla., many trees of *P. taeda* are badly infected with this *Peridermium*, and in almost every instance the diseased branches terminate in hexenbesen. Occasionally the fusiform swellings are very long, ranging from several inches to over 4 feet in length. The largest swellings are found on the trunks of trees 3 to 6 inches in diameter. Those on the 3-needle pines often originate near the extremity of a branch, and, as the side branches develop, the fungus invades them, producing an enlargement of the base of each branch. In such cases a continuous swelling is formed, extending in both directions on the main branch and to the adjacent side branches.

The junior writer collected on March 6, 1914, sporulating specimens of *Peridermium fusiforme* on *Pinus taeda* (F. P. 15138)<sup>1</sup> near Gainesville, Fla., associated directly with the young leaves of *Quercus nigra*. On March 23 he found *Cronartium cerebrum* on *Q. nigra* (F. P. 15170) and on *Q. phellos* in direct contiguity with *Peridermium fusiforme* on *P. taeda* (F. P. 15177) near Brooksville, Fla. Inoculations were made by the senior writer with the aëciospores of the first collection (F. P. 15138) on oaks (*Quercus* spp.) on March 10, 1914, in the pathological greenhouses at Washington, D. C. On April 3 the telia of *C. cerebrum* were present sparsely on the leaves of *Q. rubra* (F. P. 15217) and of *Q. velutina* (F. P. 15218).

Later and more abundant collections of *Peridermium fusiforme* were made by the junior author in Florida, South Carolina, and North Carolina on *Pinus serotina* and *P. taeda*. Inoculations made with aëciospores from these on several species of oaks resulted in nearly every instance in the appearance of abundant uredinia of *Cronartium cerebrum* in 7 to 10 days (Pl. XI, fig. 4) and of numerous telia in 15 to 21 days from the time of inoculation. All control plants in every set remained free from infection. The following species of oaks were most abundantly infected with the *Cronartium* from inoculations made with *Peridermium fusiforme*: *Quercus californica* (F. P. 15316), *Q. digitata* (F. P. 15300), *Q. gambelii* (F. P. 15287), *Q. imbricaria* (F. P. 15286), *Q. lobata* (F. P. 15299), *Q. michauxii* (F. P. 15294), *Q. phellos* (F. P. 15278), and *Q. rubra* (F. P. 15297). The following species of trees were infected less abundantly: *Castanopsis chrysophylla* (F. P. 15334), *Quercus alba* (F. P. 15309), *Q. bicolor* (F. P. 15331), *Q. emoryi* (F. P. 15318), *Q. velutina* (F. P. 15330), and *Q. virginiana* (F. P. 15317).

The uredinia, telia, and sporidia of the *Cronartium* resulting from inoculations with the aëciospores of *Peridermium fusiforme* differ in no essential feature from those obtained by inoculating the same species of oaks with the aëciospores of *P. cerebrum*; in fact they can not be distinguished morphologically from the latter (Pl. XI, fig. 3).

<sup>1</sup>The numbers in parentheses refer to specimens in the collections for study in the Office of Investigations in Forest Pathology.

The chief difference between these two species of *Peridermium*, according to the original description of each, appears to be the formation of globoid swellings on the host tree attacked by *Peridermium cerebrum* (Pl. XI, fig. 1) and of fusiform swellings by *P. fusiforme* (Pl. XI, fig. 2). Both species have the cerebroid arrangement of the æcia, while their æciospores and peridial cells agree closely in size, color, and shape.

From a series of field observations made by the senior writer during the last four years it is established that in case of the swellings of *Peridermium cerebrum* on *Pinus virginiana* the pycnia precede the æcia 12 months, instead of preceding them during the same spring. In other words, the pycnia and æcia occur during alternate years, and two years is the time required for a life cycle of all forms of spores of the rust. Usually only pycnia or only æcia are found on a gall during the same season, but occasionally both are found on different parts of the same swelling. In such cases the part bearing pycnia does not bear æcia till the following spring and vice versa. In no case have both pycnia and æcia been found at the same time, the one succeeding the other during the same season on the same portion of the surface of a swelling.

The junior writer recently noted pycnia on the swellings caused by *Peridermium fusiforme* on pines in Florida. They were noted as occurring on separate swellings from those bearing æcia, indicating the same alternation as in *P. cerebrum*.

The field and cultural data here given prove conclusively that *Peridermium fusiforme* and *P. cerebrum* are both æcial stages of the same fungus, *Cronartium cerebrum*, and are not even sufficiently differentiated to constitute separate races.

Arthur and Kern<sup>1</sup> also make *Peridermium fusiforme* a synonym of *P. cerebrum*. They state that this conclusion was reached from cultures made by them in 1913; however, their cultural data were not given in this article.

<sup>1</sup> Arthur, J. C., Kern, F. D. North American species of *Peridermium* on pine. In *Mycologia*, v. 6, no. 3, p. 133-138.

# PLATE XI

Fig. 1.—A globose swelling formed by *Peridermium cerebrum* on *Pinus echinata*, showing the cerebriform arrangement of the æcia.

Fig. 2.—A fusiform swelling caused by *Peridermium fusiforme* on *Pinus taeda*, showing a similar cerebriform arrangement of the æcia.

Fig. 3.—A leaf of *Quercus rubra* bearing the telia of *Cronartium cerebrum* from an inoculation with æciospores of *Peridermium cerebrum* on *Pinus virginiana*.

Fig. 4.—A leaf of *Quercus phellos* bearing the uredinia of *Cronartium cerebrum* from an inoculation with the æciospores of *Peridermium fusiforme*.

